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National Toxicology Program
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NTP Technical Report on the Toxicity Studies of Perfluoroalkyl Sulfonates (Perfluorobutane Sulfonic Acid, Perfluorohexane Sulfonate Potassium Salt, and Perfluorooctane Sulfonic Acid) Administered by Gavage to Sprague Dawley (Hsd:Sprague Dawley SD) Rats

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Foreword

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA). Established in 1978, the NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

The Toxicity Study Report series began in 1991. The studies described in the Toxicity Study Report series are designed and conducted to characterize and evaluate the toxicologic potential of selected substances in laboratory animals (usually two species, rats and mice). Substances selected for NTP toxicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. The interpretive conclusions presented in the Toxicity Study Reports are based only on the results of these NTP studies. Extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection per se is not an indicator of a substance's toxic potential.

The NTP conducts its studies in compliance with its laboratory health and safety guidelines and FDA Good Laboratory Practice Regulations and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. Studies are subjected to retrospective quality assurance audits before being presented for public review.

NTP Toxicity Study Reports are indexed in National Center for Biotechnology Information (NCBI) Bookshelf and are available free of charge electronically on the NTP website (http://ntp.niehs.nih.gov). Additional information regarding this study may be requested through Central Data Management (CDM) at cdm@niehs.nih.gov. Toxicity data are available through NTP's Chemical Effects in Biological Systems (CEBS) database.

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The draft *NTP Technical Report on the Toxicity Studies of Perfluoroalkyl Sulfonates (Perfluorobutane Sulfonic Acid, Perfluorohexane Sulfonate Potassium Salt, and Perfluorooctane Sulfonic Acid) Administered by Gavage to Sprague Dawley (Hsd:Sprague Dawley SD) Rats* was evaluated by the reviewers listed below. These reviewers served as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determined if the design and conditions of these NTP studies were appropriate and ensured that this NTP Toxicity Study Report presented the experimental results and conclusions fully and clearly.

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Abstract

Widespread exposure to several per/polyfluorinated alkyl substances (PFAS) is associated with a variety of toxicities that include liver and endocrine toxicity. The National Toxicology Program (NTP) conducted 28-day toxicity studies in male and female Sprague Dawley (Hsd:Sprague Dawley SD) rats (n = 10/dose; five doses per chemical) to compare the toxicities of seven PFAS (three sulfonic acids or salt: perfluorobutane sulfonic acid [PFBS], perfluorohexane sulfonate potassium salt [PFHxSK], and perfluorooctane sulfonic acid [PFOS], and four carboxylates) via gavage in deionized water with 20% Tween® 80. This report describes the studies for the two sulfonic acids (PFBS and PFOS) and salt (PFHxSK); a companion report (NTP Toxicity Study Report 97) describes the studies for the PFAS carboxylates. Doses were 0 to 1,000 mg/kg/day for PFBS, 0 to 10 mg/kg/day for PFHxSK males, 0 to 50 mg/kg/day for PFHxSK females, and 0 to 5 mg/kg/day for PFOS.

A peroxisome proliferator-activated receptor alpha (PPARα) agonist (Wyeth-14,643) was used for qualitative comparison to the PFAS evaluated (0 to 25 mg/kg/day). These studies evaluated clinical pathology, thyroid hormones, liver expression of PPARα- (*Cyp4a1, Acox1)* and constitutive androstane receptor (CAR)-related genes (*Cyp2b1, Cyp2b2*), liver acyl-CoA oxidase enzyme activity (males only), plasma and liver (males only) parent compound concentrations, and histopathology.

There was no effect on survival in PFOS or PFHxSK rats, but reduced survival was observed in the PFBS rats. Lower body weights were observed in PFBS rats and to a lesser extent in PFOS rats. Plasma and liver concentrations normalized to dose were the highest in male and female PFOS rats and the lowest in PFBS rats with apparent sex differences in plasma concentrations observed in PFHxSK rats. Findings that occurred in two or more PFAS were increased liver weights (absolute and relative to body weight), increased *Cyp4a1, Acox1, Cyp2b1, Cyp2b2* expression, increased acyl-CoA oxidase activity. Several clinical chemistry endpoints were altered in PFBS and PFOS including increased liver enzyme activities; increased bile acid and direct bilirubin concentrations; and decreased globulin, cholesterol, and triglyceride concentrations. In PFHxSK males, globulin, cholesterol, and triglyceride concentrations were decreased. Reticulocyte counts were decreased in all but the PFHxSK females. Histopathologic findings included hepatocellular hypertrophy and/or cytoplasmic alteration, bone marrow hypocellularity, and lesions of the nose. Decreases in thyroid hormones were present across these chemicals and occurred at almost all doses administered, but thyroid stimulating hormone did not increase in response.

In bacterial mutagenicity tests, PFBS was equivocal in *Salmonella typhimurium* strain TA98 with or without exogenous metabolic activation; all other results for PFBS and PFOS were negative. In vivo, no increases in micronucleated reticulocytes were observed in male or female rats administered PFBS, PFHxSK, or Wyeth-14,643. In male rats administered PFOS in vivo, no increases were observed; an equivocal result was observed in female rats administered PFOS.

In general, the effects in male and female rats administered PFHxSK were of lower magnitude (e.g., liver or clinical pathology findings) or not apparent compared to the effects in rats exposed to PFBS and PFOS. This corresponded, to some degree, with limited to no increases in liver *Acox1* and *Cyp* gene expression changes. Several of the effects observed in the liver were also observed in rats administered Wyeth-14,643, but effects observed outside the liver by the PFAS were not observed with Wyeth-14,643. These data provide a basis for comparisons across the PFAS class, either using external (e.g., mol/kg/day) or internal (e.g., plasma μM) dose.

Summary of Findings Considered to Be Toxicologically Relevant in Sprague Dawley (Hsd:Sprague Dawley SD) Rats Administered Perfluoroalkyl Sulfonates by Gavage for 28 Days

	PFBS		PFHxSK		PFOS		WY	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Doses in Deionized Water with Tween® 80 (mg/kg/day)	0–1,000[a]	0–1,000[a]	0–10	0–50	0–5	0–5	0–25	0–25
Survival Rates	↓	↓	No effect	No effect	No effect	No effect	No effect	No effect
Body Weights	↓	↓	No effect	No effect	↓	↓	↓	No effect
Organ Weights								
R. Adrenal Gland								
Absolute	No effect	No effect	↓	↑	↓	No effect	No effect	No effect
Relative	No effect	No effect	↓	↑	No effect	No effect	No effect	No effect
Heart								
Absolute	↓	↓	No effect	No effect	No effect	↓	↓	No effect
Relative	↓	No effect	No effect	No effect	No effect	↓	No effect	No effect
R. Kidney								
Absolute	↑	No effect	No effect	No effect	No effect	No effect	↑	↑
Relative	↑	↑	↑	No effect	No effect	No effect	↑	↑
Liver								
Absolute	↑	↑	↑	↑	↑	↑	↑	↑
Relative	↑	↑	↑	↑	↑	↑	↑	↑
Lung								
Absolute	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Relative	No effect	No effect	No effect	No effect	No effect	↑	No effect	No effect
Spleen								
Absolute	No effect	↓	No effect	No effect	↓	No effect	↓	No effect
Relative	No effect	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Thymus								

	PFBS		PFHxSK		PFOS		WY	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Absolute	↓	↓	No effect	No effect	↓	↓	No effect	No effect
Relative	↓	No effect	No effect	No effect	↓	No effect	No effect	No effect
Hematology								
Erythrocytes	↓	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Hemoglobin	↓	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Hematocrit	↓	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Reticulocytes	↓	↓	↓	No effect	↓	↓	No effect	No effect
Leukocytes	No effect	No effect	No effect	No effect	↓	No effect	No effect	No effect
S. Neutrophils	No effect	No effect	No effect	No effect	↓	No effect	No effect	No effect
Clinical Chemistry								
Total Protein	↓	No effect	No effect	No effect	No effect	↑	↓	↑
Albumin	No effect	No effect	No effect	No effect	↑	↑	↑	↑
Globulin	↓	No effect	↓	No effect	↓	No effect	↓	↓
Albumin/Globulin Ratio	↑	No effect	↑	No effect	↑	↑	↑	↑
Total Bilirubin	No effect	↑	No effect	No effect	No effect	↑	No effect	No effect
Direct Bilirubin	↑	↑	No effect	No effect	↑	↑	↑	No effect
Cholesterol	↓	↓	↓	No effect	↓	↓	No Effect	No effect
Triglycerides	↓	No effect	↓	No effect	↓	↓	↑	No effect
Alanine Aminotransferase	↑	↑	No effect	No effect	↑	↑	↑	↑
Alkaline Phosphatase	↑	↑	No effect	No effect	↑	↑	↑	↑
Aspartate Aminotransferase	No effect	↑	No effect	No effect	↑	No effect	↑	↑
Sorbitol Dehydrogenase	↑	No effect	No effect	No effect	No effect	No effect	↑	↑
Bile Acid	↑	↑	No effect	No effect	↑	↑	↑	No effect
Thyroid Stimulating Hormone	No effect	No effect	No effect	No effect	No effect	No effect	↓	↑
Total Thyroxine	↓	↓	↓	↓	↓	↓	↓	No effect

	PFBS		PFHxSK		PFOS		WY	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Free Thyroxine	↓	↓	↓	↓	↓	↓	↓	No effect
Total Triiodothyronine	↓	↓	↓	No effect	↓	↓	No effect	↑
Testosterone	No effect	No effect	No effect	No effect	No effect	No effect	↓	No effect
Gene Expression								
Acox1	↑	↑	↑	No effect	↑	↑	↑	↑
Cyp4a1	↑	↑	↑	No effect	↑	↑	↑	↑
Cyp2b1	↑	↑	↑	↑	↑	↑	↑	↑
Cyp2b2	↑	↑	↑	↑	↑	↑	↑	↑
Acyl-CoA Oxidase	↑	NA	↑	NA	↑	NA	↑	NA
Reproductive Toxicity								
Altered Estrous Cyclicity	NA	Yes	NA	No effect	NA	Yes	NA	Yes
Altered Sperm Parameters	No effect	NA	No effect	NA	No effect	NA	Yes	NA
Nonneoplastic Effects								
Liver:								
Hepatocyte, Cytoplasmic Alteration	↑	↑	No effect	No effect	No effect	↑	↑	↑
Hepatocyte, Hypertrophy	↑	↑	↑	No effect	↑	↑	↑	↑
Hepatocyte, Vacuolization Cytoplasmic	No effect	No effect	No effect	No effect	↑	No effect	No effect	No effect
Necrosis	↑	↑	No effect	No effect	No effect	No effect	No effect	No effect
Bone Marrow:								
Hypocellularity	↑	↑	No effect	No effect	↑	↑	No effect	No effect
Kidney:								
Papilla, Necrosis	↑	↑	No effect	No effect	No effect	No effect	No effect	No effect
Nose:								
Olfactory Epithelium, Degeneration	↑	↑	No effect	↑	No effect	No effect	No effect	No effect

	PFBS		PFHxSK		PFOS		WY	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Olfactory Epithelium, Hyperplasia	↑	↑	No effect	↑	No effect	No effect	No effect	No effect
Olfactory Epithelium, Inflammation, Suppurative	↑	↑	No effect	↑	No effect	No effect	No effect	No effect
Olfactory Epithelium, Necrosis	↑	↑	No effect	No effect	No effect	No effect	No effect	No effect
<u>Spleen:</u>								
Extramedullary Hematopoiesis, Decreased	No effect	No effect	No effect	No effect	↑	↑	No effect	No effect
<u>Stomach, Forestomach:</u>								
Epithelium, Hyperplasia	↑	No effect	No effect	No effect	No effect	No effect	No effect	No effect
<u>Thymus:</u>								
Atrophy	↑	↑	No effect	No effect	No effect	No effect	No effect	No effect
Genetic Toxicology								
Bacterial Gene Mutations	Equivocal in *S. typhimurium* strain TA98, with and without S9 mix; negative in TA100 and *E. coli* strain WP2 *uvr*A/pKM101, with and without S9		Not tested		Negative in *S. typhimurium* strains TA98 and TA100 and *E. coli* strain WP2 *uvr*A/pKM101, with and without S9		Not tested	
Micronucleated Erythrocytes								
Rat Peripheral Blood In Vivo	Negative	Negative	Negative	Negative	Negative	Equivocal	Negative	Negative

PFBS = perfluorobutane sulfonic acid; PFHxSK = perfluorohexane sulfonic acid potassium salt; PFOS = perfluorooctane sulfonic acid; WY = Wyeth-14,643; NA = not applicable; reproductive hazard calls were not considered appropriate.
[a]One-half the dose was administered daily.

Overview

The U.S. Environmental Protection Agency nominated the per/polyfluorinated alkyl substances (PFAS) class to the National Toxicology Program (NTP) for a variety of toxicity assessments. A major component of this nomination was a class toxicity evaluation of PFAS through in vitro or in vivo studies. The studies presented in this Toxicity Study Report are part of the in vivo class evaluation, which evaluated a suite of seven PFAS chemicals consisting of three alkyl sulfonates and four carboxylates of varying chain length (see list below). Measurement of plasma and liver levels of each chemical was conducted to allow for the correlation of toxicities to internal exposure parameters. An agonist of peroxisome proliferator-activated receptor alpha (PPARα), Wyeth-14,643, was included for qualitative comparisons to the PFAS tested because several PFAS are known to activate the PPARα pathway. These studies are divided into two reports: those focused on the alkyl sulfonate subclass are presented in this report and those focused on the carboxylates are presented in NTP Toxicity Study Report 97.

Other PFAS studies that were conducted with this nomination included an assessment of the contribution of perinatal exposure to perfluorooctanoic acid (PFOA) chronic toxicity and carcinogenicity in rats; an evaluation of the toxicokinetics of seven PFAS chemicals after a single dose in rats; and in vitro class evaluations of potential neurotoxicity, mitochondrial toxicity, and immunotoxicity with a follow-up in vivo immune toxicity study on perfluorodecanoic acid. As the PFAS class continues to expand with new uses and replacements, NTP continues to assess the potential toxicity of these chemicals through a variety of methods, including in silico, in vitro, and in vivo studies.

Perfluoroalkyl Substances Studied in Toxicity Reports 96 and 97

Chemical	CAS No.	Abbreviation	Toxicity Study Report
Perfluorobutane Sulfonic Acid	375-73-5	PFBS	96
Perfluorohexane Sulfonate Potassium Salt	3871-99-6	PFHxSK	96
Perfluorooctane Sulfonic Acid	1763-23-1	PFOS	96
Perfluorohexanoic Acid	307-24-4	PFHxA	97
Perfluorooctanoic Acid	335-67-1	PFOA	97
Perfluorononanoic Acid	375-95-1	PFNA	97
Perfluorodecanoic Acid	335-76-2	PFDA	97
Wyeth-14,643	50892-23-4	WY	96/97

Due to the comparative nature of the studies and concerns of variable kinetics across the class, NTP conducted toxicokinetic studies in the Sprague Dawley rat to understand the relationship of external dose to internal dose. A maximum tolerated daily dose was identified and four lower doses were selected on the basis of the resulting data and supporting kinetic and toxicity information from the literature. PFAS that displayed rapid elimination due to chain length or sex differences were administered at higher doses compared to PFAS with longer half-lives. Doses were administered twice daily for PFBS and PFHxA. The following table lists doses in mg/kg/day and μmol/kg/day for comparison for the chemicals discussed in both Toxicity Study Reports.

Doses Administered to Sprague Dawley Rats in the Gavage Studies of Perfluoroalkyl Substances and Wyeth-14,643

PFAS	Sex	Dose (mg/kg/day)	Micromolar Dose (μmol/kg/day)
PFBS	M/F	0, 62.6, 125, 250, 500, 1,000*	0, 208.6, 416.5, 833.1, 1,666, 3,332*
PFHxSK	M	0, 0.625, 1.25, 2.5, 5, 10	0, 1.4, 2.9, 5.7, 11.4, 22.8
	F	0, 3.12, 6.25, 12.5, 25, 50	0, 7.1, 14.3, 28.5, 57.1, 114.1
PFOS	M/F	0, 0.312, 0.625, 1.25, 2.5, 5	0, 0.6, 1.2, 2.5, 5.0, 10.0
PFHxA	M/F	0, 62.6, 125, 250, 500, 1,000*	0, 199.3, 398.0, 796.0, 1,592, 3,184*
PFOA	M	0, 0.625, 1.25, 2.5, 5, 10	0, 1.5, 3.0, 6.0, 12.1, 24.2
	F	0, 6.25, 12.5, 25, 50, 100	0, 15.1, 30.2, 60.4, 120.8, 241.5
PFNA	M	0, 0.625, 1.25, 2.5, 5, 10	0, 1.3, 2.7, 5.4, 10.8, 21.5
	F	0, 1.56, 3.12, 6.25, 12.5, 25	0, 3.4, 6.7, 13.5, 26.9, 53.9
PFDA	M/F	0, 0.156, 0.312, 0.625, 1.25, 2.5	0, 0.3, 0.6, 1.2, 2.4, 4.9
WY	M/F	0, 6.25, 12.5, 25	0, 19.3, 38.6, 77.2

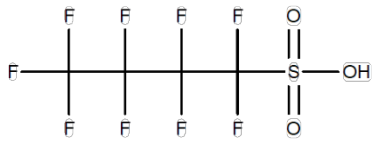
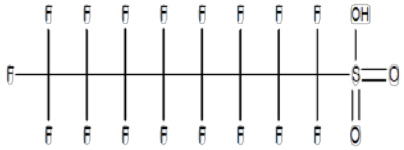
PFBS = perfluorobutane sulfonic acid; PFHxSK = perfluorohexane sulfonate potassium salt; PFOS = perfluorooctane sulfonic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; WY = Wyeth-14,643.
*These are the total doses per day; one-half the dose was administered twice daily.

Introduction

Chemical and Physical Properties

The per/polyfluorinated alkyl substances (PFAS) are a class of chemicals that consist of alkyl chains in which hydrogen is substituted with fluorines. The subclass of perfluoroalkyl sulfonates (or sulfonic acids) in this Toxicity Study Report consists of aliphatic sulfonates with four, six, or eight carbons (Table 1). The hydrophobic and lipophobic qualities of these chemicals make them useful in surfactants and polymers[1].

Table 1. Perfluoroalkyl Sulfonates

Chemical Chemical Formula	CAS Number	Chemical Structure	Molecular Weight
Perfluorobutane Sulfonic Acid $C_4HF_9O_3S$	375-73-5		300.1
Perfluorohexane Sulfonate Potassium Salt $C_6F_{13}O_3SK$	3871-99-6		438.2
Perfluorooctane Sulfonic Acid $C_8HF_{17}O_3S$	1763-23-1		500.1

Perfluorobutane sulfonic acid (PFBS) is a clear, colorless liquid with a boiling point of 210º to 212ºC and a vapor pressure of 0.0268 mm Hg (25ºC)[2]. Potassium perfluorohexane sulfonate (PFHxSK) is a chunky white solid with a boiling point of 238º to 239ºC and a vapor pressure of 0.0046 mm Hg (25ºC)[3]. Perfluorooctane sulfonic acid (PFOS) is an off-white powder with a boiling point of 249ºC and a vapor pressure of 0.0020 mm Hg (25ºC)[4].

Production, Use, and Human Exposure

Production of PFAS occurs through two major processes. Initially, electrochemical fluorination—developed in the 1950s (using a precursor chemical and hydrogen fluoride)—was the method for producing these sulfonates. This method produced both linear and a variety of branched isomers. Telomerization was developed later and produced linear chemicals using a perfluoroalkyl iodide precursor and tetrafluoroethylene.

These sulfonic acids were primarily used in producing surfactants and polymers that were used in carpets, clothing, and nonstick commercial products such as cookware. PFOS was voluntarily

phased out of production between 2000 and 2002[5] along with perfluorohexane sulfonate (PFHxS). PFBS is considered a replacement for these longer-chain PFAS, exhibiting a faster elimination rate. Although production of the longer-chained PFOS has ceased, precursor compounds (e.g., fluorotelomers) used in products such as fire-fighting foams may breakdown to form PFOS and PFHxS[1], contributing to ongoing exposure.

The three sulfonates evaluated in this report display a wide range of human internal exposure.

PFBS: Examination of plasma concentrations of PFBS in American Red Cross donors from 2000 through 2010 found most values were below the limit of quantitation (LOQ = 0.0250 ng/mL)[6]. Serum measurements in Taiwanese children averaged 0.48 ng/mL[7]. From 2005 to 2014, National Health and Nutrition Examination Survey (NHANES) serum measurements of PFBS have been almost entirely below the limit of detection (LOD = 0.1 ng/mL).

PFHxS: NHANES[8; 9] serum measurements of PFHxS from 1999 to 2014 range from 1 to 2 ng/mL (geometric mean). The most recent measurements suggest a slight decline compared to historical values with the 95th percentile declining from 8.7 ng/mL (1999–2000) to 5.60 ng/mL (2013–2014). Whole blood measurements in China from babies, infants, toddlers, children, and adults in 2009 showed a general increase with age (0.25 ng/mL in babies up to 0.40 ng/mL in adults)[10]. Serum plasma levels of PFHxS averaged 1 to 2 ng/mL in women from California in the 1960s[11], and these levels have decreased since the discontinuation of use in the early 2000s[11; 12], a finding consistent with the NHANES data set.

PFOS: Of the PFAS measured in sera, PFOS generally has the highest exposure levels compared to all other PFAS. Since it was phased out in the early 2000s, the levels of this chemical have continued to drop in human serum. NHANES[8; 9] data of collected human sera show a steady decrease starting from a geometric mean of 30.4 ng/mL in 1999–2000 to 9.32 ng/mL in the most recent 2013–2014 assessment. Although discontinued from use over a decade ago, the long plasma half-life of PFOS and other PFAS that are potentially metabolized to PFOS will likely result in years of continued exposure.

Regulatory Status

The U.S. Environmental Protection Agency (USEPA) is considering a human health toxicity value for PFBS and related compounds that will be based on a developmental exposure study in mice and other animal studies that show adverse kidney effects[13; 14]. The Minnesota Department of Health[15] established a guidance value of 2 ppb for PFBS in drinking water.

PFHxS, as with other PFAS chemicals, is currently listed under USEPA's UCMR3 (Third Unregulated Contaminant Monitoring Rule), which considers suspected contaminants. Production of PFHxS was discontinued at the same time as PFOS[16], and currently, there are no regulatory levels set by USEPA.

PFOS, the most prevalent of these three sulfonates, was voluntarily phased out of production between 2000 and 2002[5]. USEPA published significant new use rules requiring notification for import or manufacturing of PFOS and related sulfonates[17-19]. USEPA initially provided drinking water provisional advisories of 200 ng/L (parts per trillion) in 2009 predicated on effects on thyroid hormones in cynomolgus monkeys[20], which were updated with a lifetime health advisory of 70 ng/L PFOS in drinking water on the basis of decreased rat pup weight and survival[21].

Recently, the European Food Safety Authority (EFSA) developed tolerable weekly intakes of 13 ng/kg/week for PFOS based on human endpoints[22].

Absorption, Distribution, Metabolism, Excretion, and Toxicokinetics

Several studies have evaluated the absorption, distribution, metabolism, excretion, and toxicokinetics of these PFAS; results of these studies indicate large differences in excretion rates and are summarized in this section. Generally, the PFAS sulfonates are water soluble and most of their elimination occurs via urine. The kinetics across the class is variable depending on species, and sex in some cases. Reuptake via renal absorption has been proposed to explain the differential elimination of the class across species and sexes. The notable sex difference of PFOA elimination in rats (female greater than male elimination rate) is reduced by castration of the male thus increasing clearance of PFOA, with evidence suggesting changes in organic anion transporters (OATs) OAT2 and OAT3 as the mechanism[23]. This mechanism is assumed to apply to the other PFAS displaying similar sex differences. Physiologically based pharmacokinetic (PBPK) modeling of PFAS has included a saturable renal resorption function[24-29]. The pharmacokinetic properties were evaluated for several PFAS in the Hsd:Sprague Dawley rat, the animal model used in these 28-day toxicity studies[30-32].

PFBS: Oral administration of 30 mg potassium PFBS/kg body weight in Sprague Dawley rats led to beta half-lives of 4.7 and 7.4 hours in males and females, respectively[33]. A similar half-life was observed in males after intravenous administration (4.5 hours), whereas females displayed a shorter half-life comparatively (4.0 hours)[33]. These half-lives are consistent with short half-lives reported in Huang et al.[31] studies in Sprague Dawley rats (~5 hours in males, ~2 hours in females across multiple doses). An intravenous dose of 10 mg/kg potassium PFBS in cynomolgus monkeys demonstrated gamma half-lives of 11.3 hours in males and 83.2 hours in females, respectively. In both species, urine was a major route of elimination[33]. In human subjects (six men and one woman), the geometric mean half-life was calculated to be 25.8 days, with urine as a major route of elimination[33]. These studies show a pattern fairly consistent across PFAS of elimination rates highest in rats, lower in monkeys, and the lowest in humans.

PFHxS: A beta half-life of 29.1 days was calculated for Sprague Dawley male rats after a single dose of 10 mg/kg potassium PFHxS administered intravenously, whereas a half-life of 1.64 days was estimated for female rats[34]. An oral dose of either 1 or 20 mg/kg yielded half-lives of 25 to 27 days in female CD-1 mice and 28 to 31 days in male mice, whereas monkeys administered 10 mg/kg intravenously had half-lives of 87 or 141 days, respectively, in males and females[34]. NTP studies of PFHxSK showed a perfluorohexane sulfonic acid half-life of 15 to 18 days in male rats and 2 days in female rats after gavage administration of 4, 16, or 32 mg/kg[31].

Data from 26 retired fluorochemical production workers (24 men, 2 women) indicate the average serum half-life of PFHxS is 7.3 years (8.5 years geometric mean)[35]—with a noted, although not statistically significant, higher average in women than in men (12.8 versus 8.2 years). A recent study of 106 participants of a previously exposed population found a shorter half-life of 5.3 years[36]. Similar to PFBS, these studies show a pattern of elimination rates highest in rats, lower in monkeys, and the lowest in humans.

PFOS: Kinetics studies demonstrated half-lives of about 40 days in Sprague Dawley male rats, 60 to 70 days in female rats, and 30 to 40 days in CD-1 male and female mice after a single oral administration of 2 or 15 mg/kg in rats and 1 or 20 mg/kg in mice[37]. Huang et al.[31] studies in Hsd:Sprague Dawley SD rats showed a half-life of about 38 days in male and female rats after gavage administration of 2 or 20 mg/kg. In cynomolgus monkeys, half-lives of 132 days in males and 110 days in females were observed after intravenous administration[37].

Data from 26 retired fluorochemical production workers (24 men, 2 women) indicate the average serum half-life of PFOS is 5.4 years (4.8 years geometric mean)[35] with no noted differences between men and women. A recent study of a previously exposed population found a shorter half-life of 3.4 years[36]. Similar to the above PFAS, these studies show a pattern of elimination rates highest in rats, lower in monkeys, and the lowest in humans.

Toxicity

Experimental Animals

The literature on potential PFAS toxicity is extensive, and the following summaries focus mainly on guideline studies (primarily in rats) for comparison to the results within this report. Liver toxicity is a common finding across this class, ranging from liver hypertrophy or necrosis to alterations in liver gene expression and clinical chemistry changes. Peroxisome proliferator-activated receptor alpha (PPARα) activation—leading to increased related gene expression and enzyme activity (e.g., cytochrome P450 4A1 and acyl-CoA oxidase 1 [*Acox1*] along with related enzyme activity of acyl-CoA oxidase) by PFAS in rodents—is a mechanistic pathway with varying potency across the class depending on chain length[38-41]. Generally, longer-chain PFAS display a greater toxicity than shorter chain PFAS, and rodent species display a greater PPARα sensitivity than humans[40-42]. Gene expression profiling of the liver suggests that constitutive androstane receptor (CAR) induction is another factor in liver toxicity and enzyme alteration[43; 44].

PFBS: A 90-day study in Sprague Dawley rats administered 60, 200, or 600 mg/kg PFBS identified spleen weight decreases in males, total protein and albumin decreases in females, decreases in red blood cell parameters in males, and histologic lesions in the kidney, stomach, and nasal cavity[45]. A two-generation study in Sprague Dawley rats administered 30, 100, 300, or 1,000 mg/kg potassium PFBS identified liver lesions and no reproductive toxicity at the doses tested[46].

PFHxS: A reproductive assessment, derived from a guideline OECD 442 design, did not show reproductive or developmental effects up to 10 mg/kg/day; however, changes in clinical chemistry and liver weights were noted in F_0 males[47]. Mice administered 6.1 or 9.2 mg/kg PFHxS on postnatal day (PND) 10 displayed differential neuroprotein levels 24 hours later, primarily in the hippocampus and to a lesser degree in the cerebral cortex, but the effects were not apparent later in adult mice[48]. Another reproductive and developmental toxicity assessment in CD-1 mice noted decreases in litter size at doses of 1.0 and 3.0 mg/kg, and a number of liver-associated effects were observed in males and females[49].

PFOS: Subchronic studies in cynomolgus monkeys[20] and rats[50] observed several effects in the liver, thyroid hormones, and clinical chemistry when administered up to 0.75 mg/kg/day in

monkeys or 20 ppm in the diet to rats. A two-generation study in rats administered doses up to 3.2 mg/kg/day observed reduced pup survival and lower body weights at 1.6 and 3.2 mg/kg/day[51]. Gestational exposure in mice and rats up to 10 or 20 mg/kg/day, respectively, led to reduced maternal weights, with fetal malformations at the highest doses[52] and neonatal death at the higher doses[53]. Immune toxicity studies have observed effects from PFOS exposure, which may be independent of PPARα activation[54].

Humans

Several epidemiological studies have associated PFAS exposure with disease, with many studies focused on PFOA exposure. Because coexposure to several PFAS is likely, it is difficult to ascertain the contribution of individual PFAS. PFAS have been associated with immune modulation[55], endocrine disruption[56; 57], sperm quality[58], and clinical chemistry effects[59-61] among other findings. Elevated serum PFOA levels during development has been associated with a reduced antibody response (postvaccination) as observed in the Norwegian and Faroe Island birth cohort studies[62-64] and a cross-sectional study of adolescents using NHANES data[65]. NTP[66] conducted a systematic review of immune effects and concluded that PFOA is presumed to be an immune hazard due to evidence from human and animal studies. ATSDR[67] and the C8 panel reports[68-71] found associations between exposure and reproductive parameters. EFSA considered changes in clinical chemistry parameters as critical effects in their assessment[22].

Carcinogenicity

Experimental Animals

PFOS (potassium PFOS) was evaluated in a two-year bioassay in Sprague Dawley rats via diet up to 20 ppm[72]. Increases in hepatocellular adenomas in males and females were observed, whereas an increase in thyroid follicular cell adenoma was observed in a recovery group initially exposed to 20 ppm. Animal carcinogenicity studies for PFBS and PFHxS are not available.

Humans

As with the toxicity studies, most epidemiological studies have focused on PFOA with similar impediments of coexposure to other PFAS. An International Agency for Research on Cancer[73] (IARC) evaluation of PFOA classified the chemical as possibly carcinogenic to humans (Group 2B) on the basis of limited evidence in experimental animals and limited evidence in humans of positive associations with testis and kidney cancers.

Genetic Toxicity

Of the three PFAS evaluated by NTP, the published genetic toxicity data have been focused primarily on PFOS, and with one exception, the genotoxicity data for this chemical derive from in vitro assays using human or rodent cells. Thus far, the small number of in vitro studies that are available for review have reported conflicting results for the genotoxicity of PFOS. Although one study reported positive results for the genotoxicity of this chemical in vivo, a suboptimal time point was used for assessment. Additional research is needed to determine whether PFOS, as well as PFBS and PFHxS, have a potential for inducing genetic damage in vivo and in vitro.

Significant increases in DNA damage were detected using the comet assay in HepG2 cells exposed to PFOS at concentrations ranging from 0.2 to 20 μM for 24 hours[74]. However, negative comet assay results were reported in HepG2 cells exposed to PFOS at concentrations ranging from 5 to 300 μM for 1 or 24 hours[75]. Furthermore, no induction of micronuclei was observed in HepG2 cells exposed to the same concentrations of PFOS that were used for the 24-hour comet assay[75]. One notable difference between experiments conducted by Wielsøe et al.[74] and those conducted by Florentin et al.[75] was the final concentration of dimethyl sulfoxide (DMSO) (0.04% versus 2.5%, respectively). DMSO is a known scavenger of free radicals and can protect DNA from oxidative damage[76]. It has been hypothesized in some studies reviewed here that perfluorinated chemicals such as PFOS may exert genotoxic effects indirectly through generation of oxygen radicals[74; 75; 77]; therefore, excess DMSO may potentially inhibit the genotoxic effects of perfluorinated chemicals. Additionally, PFOS was negative in a micronucleus assay employing hamster lung V79 cells exposed to 12.5 μg/mL (25 μM; 1% DMSO) for 4 hours in the presence of S9 mix[78]; the authors did not test additional dose levels nor did they provide a rationale for the dose they chose to test. Biochemical experiments suggest that PFOS inhibits the activity of DNA polymerase β by interacting with the enzyme[79].

In a single reported in vivo study, significant increases in DNA damage, as measured by the comet assay, and significant, dose-dependent increases in micronuclei were observed in bone marrow cells of female Wistar rats exposed to 0.6, 1.25, or 2.5 mg/kg PFOS by oral gavage once every 2 days for 4 weeks[77]. However, the timing of the tissue sampling (30 hours after exposure) in relation to the treatment time was longer than what is appropriate for these assays, making these results difficult to understand.

In a single report identified for another perfluorinated compound, perfluorohexane sulfonic acid, significant increases in DNA damage were detected using the comet assay in HepG2 cells exposed to concentrations of the chemical ranging from 0.2 to 20 μM for 24 hours[74].

Study Rationale

USEPA nominated the PFAS class for comparative toxicity evaluation to understand the relative toxicity of individual members of the class. Chemicals were selected by comparing newer replacement chemicals (perfluorohexanoic acid, and PFBS) that are shorter in chain length to the older, longer-chained PFAS (perfluorooctanoic acid and PFOS) in addition to other environmental PFAS of varying exposure levels (PFHxS, perfluorononanoic acid, and perfluorodecanoic acid). The 28-day toxicity study design was used to evaluate multiple endpoints for each of these chemicals for comparison. Plasma concentrations of each PFAS were measured due to the wide range in kinetics of the individual chemicals across species and sexes. A PPARα positive control study (Wyeth-14,643) was included to understand which PFAS toxicological findings were consistent with this mechanism of toxicity and which appear to be independent of this mechanism.

This study is part of a larger NTP program to evaluate PFAS chemicals, which has included an assessment of the contribution of perinatal exposure to PFOA chronic toxicity and carcinogenicity in rats[80]; evaluation of the toxicokinetics of seven PFAS chemicals after a single dose in rats[32]; and in vitro class evaluations of potential neurotoxicity[81], mitochondrial toxicity[82], and immunotoxicity[83; 84] with a follow-up in vivo immune toxicity study on perfluorodecanoic acid[85].

Materials and Methods

Procurement and Characterization

Perfluoroalkyl Sulfonates

Perfluorobutane sulfonic acid (PFBS) was obtained from Sigma Aldrich (St. Louis, MO) in one lot (15414TE), perfluorohexane sulfonic acid potassium salt (PFHxSK) was obtained from Interchim (Montlucon Cedex, France) in one lot (230002), and perflourooctane sulfonic acid (PFOS) was obtained from Matrix Scientific (Columbia, SC) in one lot (T20G). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory and the study laboratory at Battelle (Columbus, OH) (Appendix C). Reports on analyses performed in support of the perfluorinated alkyl sulfonate studies are on file at the National Institute of Environmental Health Sciences.

Lot 15414TE (PFBS), a colorless liquid; lot 230002 (PFHxSK), a white solid; and lot T20G (PFOS), off-white particles, were identified using infrared (IR) spectroscopy, mass spectrometry (MS), and carbon-13 and fluorine-19 nuclear magnetic resonance (NMR) spectroscopy.

The purity of each perfluorinated alkyl sulfonate lot was determined by high-performance liquid chromatography (HPLC)/ion chromatography (IC) with suppressed conductivity (SC) detection, HPLC with MS detection, and proton-induced X-ray emission (PIXE) spectroscopy (measuring 72 elements from sodium to uranium). Differential scanning calorimetry (DSC) was used to determine the purity of lot T20G. In addition, Karl Fischer titration for lots 15414TE and T20G was performed by Galbraith Laboratories (Knoxville, TN).

For lot 15414TE (PFBS), Karl Fischer titration indicated 0.46% water. HPLC/IC/SC analysis indicated one major peak that was 97.7% of the total peak area and one impurity (2.3% of the total peak area). HPLC/MS analysis indicated one major peak (98.7%) and three impurities with a combined area of 1.3% of the total peak area. The overall purity of lot 15414TE was determined to be greater than 97% (Table 2).

Table 2. Purity of Chemicals in the 28-day Gavage Studies of Perfluoroalkyl Sulfonates

Chemical Name	Lot Number	Percent Purity	Number of Impurities	Percent Impurity
PFBS	15414TE	>97	1 to 3	1.3 to 2.3
PFHxSK	230002	>98	1 to 4	0.1 to 1.4
PFOS	T20G	>96	1 to 9	2.9 to 3.3

PFBS = perfluorobutane sulfonic acid; PFHxSK = perfluorohexane sulfonic acid potassium salt; PFOS = perfluorooctane sulfonic acid.

For lot 230002 (PFHxSK), HPLC/IC/SC analysis indicated one major peak that was 99.9% of the total peak area and one impurity (0.1% of the total peak area). HPLC/MS indicated one major peak (98.6% of the total peak area) and four reportable impurities with areas ≥0.1% of the total peak area. Three of the impurities were identified as PFBS (0.28% of the total peak area), perfluoropentane sulfonic acid (0.42% of the total peak area), and PFOS (0.19% of the total peak area); one impurity (0.49% of the total peak area) was not identified. PIXE spectroscopy found

lot 230002 to contain potassium consistent with the structure. The overall purity of lot 230002 was determined to be greater than 98% (Table 2).

For lot T20G (PFOS), Karl Fischer titration indicated 4.63% water. HPLC/IC/SC analysis indicated one major peak (96.7% of the total peak area) and one impurity that represented 3.3% of the total peak area. HPLC/MS indicated one major peak (68.3% of the total peak area) and nine other peaks with areas ≥0.1% of the total peak area. Three of the nine peaks were identified as PFOS isomers (cumulatively 27.7% of the total peak area). From the remaining peaks, two (cumulatively 2.5% of the total peak area) were identified as perfluoroheptane sulfonic acid isomers and one (0.4% of the total peak area) was identified as a perfluorohexane sulfonic acid isomer. PIXE spectroscopy indicated an absence of inorganic impurities. Purity estimated by DSC was approximately 98.8%. The overall purity of lot T20G was determined to be greater than 96% based on perfluorooctane sulfonic acid isomers (Table 2).

To ensure stability, the bulk chemicals were stored at refrigerated temperature (lot 15414TE), room temperature (lot 230002), or −20°C (lot T20G) in sealed amber glass containers under nitrogen (PFBS only). Reanalyses of the bulk chemicals were performed by the study laboratory, and no degradation was detected.

Wyeth-14,643

Wyeth-14,643 was obtained from ChemSyn Laboratories (Lenexa, KS) in one lot (91-314-72-07/91-314-100-33A). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory and the study laboratory at Battelle (Columbus, OH) (Appendix C).

Lot 91-314-72-07/91-314-100-33A, a white powder, was identified using infrared (IR) spectroscopy and proton and carbon-13 NMR spectroscopy. The purity of Wyeth-14,643 was determined by HPLC with ultraviolet light (UV) detection and DSC.

For lot 91-314-72-07/91-314-100-33A, HPLC/UV analysis indicated one major peak (99.4% of the total peak area) and two impurities with areas ≥0.1% of the total peak area. DSC analysis indicated an average purity of 94.3%, which was considered an indication of thermal decomposition rather than impurity of the bulk chemical. The overall purity of lot 91-314-72-07/91-314-100-33A was determined to be greater than 99%.

To ensure stability, the bulk chemical was stored at room temperature in sealed amber glass containers. Reanalyses of the bulk chemical were performed by the study laboratory, and no degradation was detected.

Tween® 80

Tween 80 (polysorbate 80) was obtained from Spectrum Laboratory Products, Inc. (Gardena, CA), and was used at a 2% concentration as the vehicle in the 28-day gavage studies. The vehicle was prepared by mixing the appropriate amount of Tween 80 with deionized water in a calibrated carboy and stirring with an overhead stirrer until all the Tween 80 was dissolved.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared by mixing PFBS, PFHxSK, PFOS, or Wyeth-14,643 with 2% Tween 80 in deionized water to give the required concentrations (Table C-2). Each

formulation pH was adjusted to between 6 to 8. The dose formulations were stored at room (PFBS and PFHxSK) or refrigerated (PFOS and Wyeth-14,643) temperatures in amber glass bottles sealed with Teflon®-lined lids for no more than 42 days.

Homogeneity studies of the 12.5 and 100 mg/mL dose formulations of PFBS and a stability study of the 12.5 mg/mL dose formulation were performed by the analytical chemistry laboratory using HPLC/IC/SC. Homogeneity was confirmed and stability was confirmed for at least 42 days for dose formulations stored in amber glass bottles sealed with Teflon-lined lids at room temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of a 0.12 mg/mL formulation and the 10 mg/mL dose formulation of PFHxSK and a stability study of a 0.12 mg/mL formulation were performed by the analytical chemistry laboratory using HPLC/IC/SC. Homogeneity was confirmed and stability was confirmed for at least 42 days for formulations stored in amber glass bottles sealed with Teflon-lined lids at room temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of a 0.06 mg/mL formulation and the 1 mg/mL dose formulation of PFOS and a stability study of a 0.06 mg/mL formulation were performed by the analytical chemistry laboratory using HPLC/IC/SC. Homogeneity was confirmed, and stability was confirmed for at least 41 days for formulations stored in amber glass bottles sealed with Teflon-lined lids at refrigerated temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of the 1.26 and 5 mg/mL dose formulations of Wyeth-14,643 and a stability study of a 1.26 mg/mL dose formulation were performed by the analytical chemistry laboratory using HPLC/UV. Homogeneity was confirmed, and stability was confirmed for at least 42 days for formulations stored in amber glass bottles sealed with Teflon-lined lids at refrigerated temperature and for at least 3 hours under simulated animal room conditions.

Analyses of the dose formulations for the 28-day studies of PFBS, PFHxSK, PFOS, and Wyeth-14,643 were conducted once for each study by the study laboratory using HPLC/IC/SC or HPLC/UV. All five dose formulations for the PFBS study were within 10% of the target concentrations (Table C-3). Animal room samples of these dose formulations were also analyzed; all six were within 10% of the target concentrations. For the PFHxSK study, all 10 dose formulations and all 10 animal room samples were within 10% of the target concentrations. For the PFOS study, all five dose formulations and all five animal room samples were within 10% of the target concentrations. For the Wyeth-14,643 study, all three dose formulations and all three of the animal room samples were within 10% of the target concentrations.

Animal Source

Male and female Sprague Dawley (Hsd:Sprague Dawley SD) rats were obtained from Harlan Laboratories, Inc., Indianapolis, IN, now part of Envigo, Inc.

Animal Welfare

Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by the AAALAC International. Studies were approved by the Battelle Columbus Operations Animal

Care and Use Committee and conducted in accordance with all relevant NIH and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines.

28-day Studies

On receipt, rats were approximately 6 to 8 weeks old. Animals were quarantined for 14 (males) or 15 (female) days for PFBS, 21 (male) or 22 (female) days for PFHxSK, 19 (male) or 20 (female) days for PFOS, and 20 (males) or 21 (females) days for Wyeth-14,643. The rats were 10 to 11 weeks old on the first day of each study. Before the studies began, five male and five female rats were randomly selected for parasite evaluation and gross observation for evidence of disease. The health of the animals was monitored during the studies according to the NTP Sentinel Animal Program (Appendix E). All results were negative.

All dose groups consisted of 10 male and 10 female rats. All test compounds were administered in deionized water with 2% Tween 80 by gavage, 7 days per week for 28 days; control animals received the vehicle only. Doses were selected on the basis of a maximum tolerated daily dose and kinetic information obtained from toxicokinetic studies. PFBS was administered twice daily at one-half the dose for total daily doses of 0, 62.6, 125, 250, 500, or 1,000 mg/kg body weight. PFHxSK was administered once daily at 0, 0.625, 1.25, 2.5, 5, or 10 mg/kg (males) or 0, 3.12, 6.25, 12.5, 25, or 50 mg/kg (females). PFOS was administered once daily at 0, 0.312, 0.625, 1.25, 2.5, or 5 mg/kg. Wyeth-14,643 was administered once daily at 0, 6.25, 12.5, or 25 mg/kg. All formulations were administered at a volume of 5 mL/kg. NTP-2000 feed and water were available ad libitum. Rats were housed five per cage and were observed twice daily. The animals were weighed, and clinical observations were recorded initially, weekly thereafter, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 3. Information on feed composition and contaminants is provided in Appendix D.

Animals were anesthetized with a 70%:30% CO_2:O_2 mixture and blood was collected from the abdominal aorta at the end of the studies for hematology, clinical chemistry, thyroid hormone, and testosterone analyses; parent compound concentration; and micronucleus assays. For each animal, blood was collected into two tubes containing K_3 EDTA and one tube devoid of an anticoagulant. An aliquot of whole blood was removed from one K_3 EDTA tube and centrifuged, and the plasma was harvested and shipped to Battelle (Columbus, OH) for internal dose analyses[30; 31]; the remaining whole blood was used for hematology analysis. The other K_3 EDTA tube was shipped on wet ice to ILS, Inc. (Durham, NC), for micronuclei analysis the day it was collected. Any remaining plasma was frozen and shipped to the NTP Frozen Tissue Bank (Durham, NC). Blood collected in tubes devoid of anticoagulant was allowed to clot, and the serum harvested for clinical chemistry and hormone analysis, except aspartate aminotransferase activity and total and direct bilirubin concentrations, which were analyzed at a later date using an aliquot of the stored frozen plasma. The hematology analyses were conducted using an Advia 120 (Bayer Diagnostics Division, Tarrytown, NY) and reagents supplied by the manufacturer. A manual hematocrit was determined with the use of a microcentrifuge. Blood smears were stained with a Romanowsky-type stain and the morphology of the leukocytes, erythrocytes and platelets reviewed. Clinical chemistry analyses (including total thyroxine) were completed using a cobas c501 Chemistry Analyzer (Roche, Indianapolis, IN); aspartate aminotransferase activity and bilirubin concentrations were analyzed on an Olympus AU400 Analyzer (Irving, TX). The remaining thyroid hormones were determined by radioimmunoassay (free thyroxine [free T4], total thyroxine [T4], and total triiodothyronine [T3] [MP Biomedicals,

Irvine, CA]; thyroid stimulating hormone [TSH] [Institute of Isotopes, Ltd., Budapest, Hungary]). Testosterone was determined by enzyme-linked immunosorbent assay (Calbiotech, Inc., Spring Valley, CA). The parameters measured are listed in Table 3.

Necropsies were performed on all rats. The right adrenal gland, heart, right kidney, liver, lung, spleen, right testis, thymus, thyroid gland, and uterus/cervix/vagina from each animal were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (except eyes, testes, epididymides and vaginal tunics were first fixed in Davidson's solution or modified Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μm, and stained with hematoxylin and eosin. Histopathologic examinations were performed on all rats. Table 3 lists the tissues and organs routinely examined.

At the end of the 28-day studies, samples were collected for sperm motility evaluations on male rats administered 0, 125, 250, or 500 mg/kg/day (PFBS); 0, 2.5, 5, or 10 mg/kg/day (PFHxSK); 0, 1.25, 2.5, or 5 mg/kg/day (PFOS), and 0, 6.25,12.5, or 25 mg/kg/day (Wyeth-14,643). It was recognized that the 28-day dosing duration would not cover the full spermatogenic cycle. The parameters evaluated are listed in Table 3. Male rats were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65°C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer. For vaginal cytology, samples were taken from females in the 0, 125, 250, and 500 mg/kg/day (PFBS); 0, 12.5, 25, and 50 mg/kg/day (PFHxSK); 0, 0.625, 1.25, and 2.5 mg/kg/day (PFOS); and 0, 6.25, 12.5, and 25 mg/kg/day (Wyeth-14,643) groups. For 16 consecutive days prior to scheduled terminal euthanasia, the vaginal vaults of the females were moistened with saline, if necessary, and samples of vaginal fluid and cells were stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus).

At the end of the PFBS, PFHxSK, and PFOS studies, 500 mg liver samples from the right anterior and posterior lobes of male rats (male rat liver responses to PFAS are generally greater compared to females) were collected and shipped to Battelle's Chemistry Technical Center for determination of parent compound concentrations. At the end of all studies, approximately 1 g of liver tissue was collected from the median lobe of male rats, homogenized, frozen, and stored until analyzed for acyl-CoA oxidase activity. From the left liver lobe, tissue samples were placed in *RNAlater*® (Ambion, Inc., Austin, TX) and stored at 2° to 8°C overnight. The *RNAlater* supernatant was then removed from the samples, and the samples were frozen at −70° ± 10°C. Samples weighing between 22.0 and 30.0 mg were added to lysis buffer, homogenized, and stored at −80° ± 10°C until RNA was isolated. RNA was extracted from the supernatant,

subjected to DNase digestion, and isolated using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Each RNA sample was analyzed for quantity and purity by ultraviolet analysis using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). All samples were evaluated for RNA integrity using an RNA 6000 Nano Chip Kit with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and then stored at −80°C ± 10°C until further processing. Total RNA (1 μg), isolated from rat tissue samples was reverse transcribed into cDNA using Qiagen RT HT First Strand Kits (Qiagen, Valencia, CA). The cDNA samples were analyzed using Qiagen RT^2 Custom Arrays in a 96-well plate format. Relative fold change was calculated within the analysis software based on the ΔΔCt method for relative quantitation. Twelve samples were run on each array plate with a single sample in each column of the plate. The eight wells in each column of the array plates contained the four genes of interest: PPARα-related genes acyl-CoA oxidase (*Acox1*) and cytochrome P450 4a1 (*Cyp4a1*), CAR-related genes cytochrome P450 2b1 (*Cyp2b1*) and cytochrome P450 2b2 (*Cyp2b2*), a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and three array plate quality controls. The parameters measured are listed in Table 3.

Quality control measurements for each sample on each qPCR array plate were evaluated to determine if the data generated from the sample were of sufficient quality prior to analysis using the web-based RT Profiler PCR Array Data Analysis software (version 3.5, SABiosciences, Valencia, CA). The controls on the qPCR array were analyzed for each sample and included a PCR positive/array reproducibility control (PPC), a reverse transcription control (RTC), and a genomic DNA contamination (GDC) control. A sample successfully passed all of these control parameters when the cycle threshold (Ct) value for the PPC was 20 ± 2, the Ct value for the GDC was greater than or equal to 35, and the Ct value of the PPC subtracted from the Ct value of the RTC was less than or equal to 5. If a sample failed the acceptance criteria for the GDC and/or RTC controls, the sample was repeated starting with the reverse transcription (cDNA synthesis) process followed by analysis on a new qPCR array plate. If a sample failed the PPC control, the cDNA sample was used to repeat the sample on a new qPCR array plate. Once all of the data from a single experiment were analyzed and collected (male or female, control animals and all dosages), the quality control data were assessed a second time after the total experimental data were uploaded into the web-based RT Array Data Analysis software (version 3.5, SABiosciences). This provided additional assurance that the data for the entire experiment passed the quality control criteria.

The relative fold change for each gene of interest (*Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2*) was calculated within the analysis software for relative quantitation based on the ΔΔCt method. All of the samples were normalized using the housekeeping gene *GAPDH* and the geometric mean of all samples in a particular group was used for normalization. The p values (based on the Student's t-test of the replicate values for each gene in the vehicle control group and dose groups) and 95% confidence intervals for each fold change value were also calculated during analysis. The threshold for determining significant differential expression of each gene was a fold change of ≥2 or ≤−2 and a p value ≤0.05.

After a review of the laboratory reports, selected histopathology slides were submitted to quality assessment (QA) pathologists for review. After the review, diagnostic discrepancies/inconsistencies were discussed and resolved with NTP pathologists. The QA pathologist also served as the Pathology Working Group (PWG) pathologist and coordinator.

Any remaining discrepancies/inconsistencies were resolved by the PWG. Final diagnoses for reviewed lesions represent a consensus of the PWG or a consensus between the study laboratory pathologist, NTP pathologist and the QA/PWG pathologist. Details of these review procedures have been described, in part, by Maronpot and Boorman[86] and Boorman et al.[87].

Table 3. Experimental Design and Materials and Methods in the 28-day Gavage Studies of Perfluoroalkyl Sulfonates

28-day Study
Study Laboratory
Battelle Columbus Operations (Columbus, OH)
Strain and Species
Sprague Dawley (Hsd:Sprague Dawley SD) rats
Animal Source
Harlan Laboratories, Inc., Indianapolis, IN (now Envigo)
Time Held Before Studies
PFBS: 14 (males) or 15 (females) days
PFHxSK: 21 (males) or 22 (females) days
PFOS: 19 (males) or 20 (females) days
WY: 20 (males) or 21 (females) days
Average Age When Studies Began
10 to 11 weeks
Date of First Dose
PFBS: January 19 (males) or 20 (females), 2012
PFHxSK: February 9 (males) or 10 (females), 2012
PFOS: January 24 (males) or 25 (females), 2012
WY: January 26 (males) or 27 (females), 2012
Duration of Dosing
7 days per week for 28 Days
Date of Last Dose
PFBS: February 15 (males) or 16 (females), 2012
PFHxSK: March 7 (males) or 8 (females), 2012
PFOS: February 20 (males) or 21 (females), 2012
WY: February 22 (males) or 23 (females), 2012
Necropsy Dates
PFBS: February 16 (males) or 17 (females), 2012
PFHxSK: March 8 (males) or 9 (females), 2012
PFOS: February 21 (males) or 22 (females), 2012
WY: February 23 (males) or 24 (females), 2012
Average Age at Necropsy
14 to 15 weeks
Size of Study Groups
10 males and 10 females

28-day Study

Method of Randomization and Identification

Animals were distributed randomly into groups of approximately equal initial mean body weights, and identified by tail tattoo.

Animals per Cage

5

Diet

NTP-2000 irradiated wafer (Zeigler Brothers, Inc., Gardners, PA), available ad libitum; changed weekly

Water

Tap water (City of Columbus municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available ad libitum

Cages

Solid polycarbonate (Lab Products, Inc., Seaford, DE); changed twice weekly

Bedding

Irradiated Sani-Chips® (P.J. Murphy Forest Products Corp., Montville, NJ), changed twice weekly

Rack Filters

Spun-bonded polyester (Snow Filtration Co., Cincinnati, OH) changed every 2 weeks

Racks

Stainless steel (Lab Products, Inc., Seaford, DE), changed every 2 weeks

Animal Room Environment

Temperature: 72° ± 3°F

Relative humidity: 50% ± 15%

Room fluorescent light: 12 hours/day

Room air changes: at least 10/hour

Doses

PFBS: 0, 62.6, 125, 250, 500, or 1,000 mg/kg per day (males and females); one-half the dose was administered twice daily approximately 6 hours apart

PFHxSK: males: 0, 0.625, 1.25, 2.5, 5, or 10 mg/kg per day; females: 0, 3.12, 6.25, 12.5, 25, or 50 mg/kg per day

PFOS: 0, 0.312, 0.625, 1.25, 2.5, or 5 mg/kg per day (males and females)

WY: 0, 6.25, 12.5, or 25 mg/kg per day (males and females)

All doses were administered in deionized water with 2% Tween® 80 by gavage (dosing volume, 5 mL/kg)

Type and Frequency of Observation

Observed twice daily; animals were weighed and clinical findings were recorded on day 1, weekly thereafter, and at the end of the study.

Method of Euthanasia

Anesthetization with CO_2:O_2 (70%:30%) followed by exsanguination by severing the abdominal aorta

Necropsy

Necropsies were performed on all rats. Organs weighed were right adrenal gland, heart, right kidney, liver, lung, spleen, right testis, thymus, thyroid gland, and uterus/cervix/vagina.

Clinical Pathology

Blood was collected from the abdominal aorta of all rats at the end of the studies for hematology and clinical chemistry.

28-day Study

Hematology: hematocrit; manual hematocrit, hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials, blood smear morphological evaluation

Clinical chemistry: urea nitrogen, creatinine, glucose, total protein, albumin, globulin, total, direct, and indirect bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, cholesterol, triglycerides, bile acids, total triiodothyronine, thyroid stimulating hormone, total and free thyroxine, and testosterone

Histopathology

PFBS: Complete histopathology was performed on rats in the 0, 500, and 1,000 mg/kg per day groups. In addition to gross lesions and tissue masses, the following tissues were examined to a no-effect level: adrenal gland, bone with marrow, brain, clitoral gland, epididymis, esophagus, eyes, Harderian gland, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), lung, lymph nodes (mandibular and mesenteric), mammary gland, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, seminal vesicle, skin, stomach (forestomach and glandular), trachea, urinary bladder, and uterus. The bone marrow, kidney, liver, nose, ovary, pancreas, spleen, testes, thymus, and thyroid gland were examined in all dose groups.

PFHxSK: Complete histopathology was performed on rats in the 0, 10 (males), and 50 (females) mg/kg per day groups. The tissues examined to a no-effect level were the same as in the PFBS study but also included thymus. The bone marrow, kidney, liver, nose, ovary, pancreas, spleen, testes, and thyroid gland were examined in all dose groups.

PFOS: Complete histopathology was performed on rats in the 0 and 5 mg/kg per day groups. The tissues examined to a no-effect level and those examined in all dose groups were the same as in the PFHxSK study.

WY: Complete histopathology was performed on rats in the 0 and 25 mg/kg per day groups. The tissues examined to a no-effect level and those examined in all dose groups were the same as in the PFHxSK study.

Reproductive Parameters

PFBS: Blood was collected from the abdominal aorta of all rats at the end of the study and analyzed for testosterone levels. Spermatid and sperm samples were collected from 0, 125, 250, and 500 mg/kg per day males at study termination. The following parameters were evaluated: spermatid heads per testis and per gram testis, and epididymal spermatozoal motility and concentration. The left cauda, left epididymis, and left testis were weighed. Vaginal samples were collected for 16 consecutive days prior to the end of the study from females in the 0, 125, 250, and 500 mg/kg per day groups for vaginal cytology evaluations. The percentage of time spent in the various estrous cycle stages and estrous cycle length were evaluated.

PFHxSK: Same as PFBS study except sperm samples were collected from 0, 2.5, 5, and 10 mg/kg per day males, and vaginal samples were collected from 0, 12.5, 25, and 50 mg/kg per day females.

PFOS: Same as PFBS study except sperm samples were collected from 0, 1.25, 2.5, and 5 mg/kg per day males, and vaginal samples were collected from 0, 0.625, 1.25, and 2.5 mg/kg per day females.

WY: Same as PFBS study except sperm and vaginal samples were collected from rats in all dose groups.

Internal Dose Assessment

Approximately 24 hours after the last dose in the PFBS, PFHxSK, and PFOS studies, parent compound concentrations were determined in plasma from blood collected from the abdominal aorta of all rats and in liver samples from the right anterior and posterior lobes of male rats 24 hours after the last dose.

Liver Enzyme Levels and Gene Expression

Samples were collected from the median liver lobe of surviving male rats at study termination for determination of acyl-CoA oxidase activity. Samples were collected from the left liver lobe of all surviving rats at study termination for determination of *Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2*.

PFBS = perfluorobutane sulfonic acid; PFHxSK = perfluorohexane sulfonic acid potassium salt; PFOS = perfluorooctane sulfonic acid; WY = Wyeth-14,643.

Statistical Methods

Calculation and Analysis of Lesion Incidences

The incidences of lesions are presented in this report and in the CEBS database (https://manticore.niehs.nih.gov/cebssearch) as the numbers of animals bearing such lesions at a specific anatomic site and as the numbers of animals with that site examined microscopically. The Fisher exact test[88], a procedure based on the overall proportion of affected animals, was used to determine significance.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dose and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett[89] and Williams[90; 91]. Hematology, clinical chemistry, hormones, estrous cycle length, number of estrous cycles, parent compound, spermatid, epididymal spermatozoal, hepatic enzymes, and hepatic gene expression data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley[92] (as modified by Williams[93]) and Dunn[94]. Jonckheere's test[95] was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey[96] were examined by NTP personnel, and implausible values were eliminated from the analysis. Tests for extended periods of estrus, diestrus, metestrus, and proestrus, as well as skipped estrus and skipped diestrus, were constructed based on a Markov chain model proposed by Girard and Sager[97]. For each dose group, a transition probability matrix was estimated for transitions among the proestrus, estrus, metestrus, and diestrus stages, with provision for extended stays within each stage as well as for skipping estrus or diestrus within a cycle. Equality of transition matrices among dose groups and between the control group and each dose group was tested using chi-square statistics.

Quality Assurance Methods

The 28-day studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations[98]. In addition, as records from the 28-day studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent QA contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Toxicity Study Report. Audit procedures and findings are presented in the reports and are on file at National Institute of Environmental Health Sciences. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this report.

Genetic Toxicology

Bacterial Mutagenicity Test Protocol

The test articles were coded prior to testing. Testing was conducted as reported by Zeiger et al.[99], with some modifications. PFBS and PFOS were incubated with the bacterial tester strains (*Salmonella typhimurium* strains TA98 and TA100 and *Escherichia coli* strain WP2 *uvr*A pKM101) either in buffer or S9 mix (metabolic activation enzymes and cofactors from livers of phenobarbital/benzoflavone-induced male Sprague Dawley rats) for 20 minutes at 37°C. Top agar supplemented with L-histidine or L-tryptophan (for the *E. coli* strain only) and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine- or tryptophan-independent mutant colonies arising on these plates were counted using an automatic colony counter following incubation for 2 days at 37ºC.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of the test compound. The highest concentration tested was limited by toxicity in the absence of S9; with S9, concentrations were either limited by toxicity or achieved the limit concentration of 10,000 μg/plate. All trials were repeated.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, not reproducible, or not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. No minimum percentage or fold increase is required for a chemical to be judged positive or weakly positive.

Rat Peripheral Blood Micronucleus Test Protocol

Blood samples (200 μL per animal) were collected at the end of the studies, stabilized in EDTA tubes and packaged on ice packs for shipment later that same day by overnight courier to ILS, Inc. (Durham, NC). Samples were kept chilled upon arrival at the laboratory the following day and were fixed within 4 hours of receipt. For each sample, 50 μL of blood were dispensed into a microcentrifuge tube containing heparin and mixed by inverting several times. A fixation tube containing ultra-cold methanol was then removed from a −80°C freezer and 180 μL of the heparinized blood sample was forcefully dispensed into the tube, rapidly mixed, and quickly transferred back to the −80°C freezer. The fixed blood samples were stored in the −80°C freezer for at least 3 days prior to flow cytometry analysis of micronucleated red blood cells.

Flow cytometry analysis was performed using MicroFlowPLUS Kit reagents (Litron Laboratories, Rochester, NY) and a Becton-Dickinson FACSCalibur™ dual-laser bench top system (BD Biosciences, San Jose, CA). The analysis was performed according to the kit's instruction manual with minimal modification[100; 101]. For five peripheral blood samples per dose group, 20,000 (±2,000) immature CD71-positive erythrocytes (also referred to as polychromatic erythrocytes [PCE]) were analyzed by flow cytometry to determine the frequency of normal and micronucleated PCE (MN-PCE). Aggregates were excluded on the basis of forward and side scatter, platelets were excluded on the basis of staining with an anti-CD61 antibody, and nucleated leukocytes were excluded on the basis of intense propidium iodide staining. Normal and micronucleated mature erythrocytes (also referred to as normochromatic erythrocytes

[NCE]) were enumerated concurrently during MN-PCE analysis, allowing for calculation of the percentage of PCE among total erythrocytes as a measure of bone marrow toxicity.

In rats, although both immature and mature erythrocytes are evaluated for presence of micronuclei concurrently, the appropriate cell population for determining the frequency of micronucleated cells is the youngest portion of the immature erythrocyte population, i.e., the fraction of immature erythrocytes that is newly emerged from the bone marrow, due to the efficiency with which the rat spleen removes micronucleated red blood cells from circulation.

Data were analyzed as described previously[102; 103]. Mean MN-PCE/1,000 RET and MN-NCE/1,000 erythrocytes, as well as %PCE, were calculated for each animal. These data are summarized in the tables as mean ± standard error of the mean. Levene's test was used to determine if variances among dose groups were equal at $p = 0.05$. When variances were equal, linear regression analysis was used to test for linear trend and Williams' test was used to evaluate pairwise differences between each dose group and the vehicle control group. When variances were unequal, nonparametric methods were used to analyze the data; Jonckheere's test was used to evaluate linear trend and Dunn's test was used to assess the significance of pairwise differences between each dose group and the vehicle control group. To maintain the overall significance level at 0.05, the trend as well as the pairwise differences from the vehicle control group were declared statistically significant if $p \leq 0.025$. A result was considered positive if the trend test was significant and if at least one dose group was significantly elevated over the vehicle control group, or if two or more dose groups were significantly increased over the corresponding vehicle control group. A response was considered equivocal if only the trend test was significant or if only a single dose group was significantly increased over the vehicle control.

Evaluation Protocol

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple samples of a chemical were tested in the same assay, and different results were obtained among these samples and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the in vitro assays have another variable that must be considered in arriving at an overall test result. In vitro assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The results presented in the Abstract of this report, Toxicity Study Report 96, represent a scientific judgment of the overall evidence for activity of the chemical in an assay.

Results

NTP evaluated all study data. Data relevant for evaluating toxicological findings are presented here. All study data are available in the NTP Chemical Effects in Biological Systems (CEBS) database: http://doi.org/10.22427/NTP-OTHER-1.

28-day Studies

Perfluorobutane Sulfonic Acid (PFBS)

Nine male rats died from day 15 to day 25 in the 1,000 mg/kg/day group, and one died due to a dosing accident on day 6 (Table 4). One 250 mg/kg/day female, one 500 mg/kg/day female, and eight 1,000 mg/kg/day females died on day 25, day 21, and from day 16 to day 27, respectively (Table 4). Except for the one dosing accident, the deaths of male and female rats were considered related to treatment, but cause of death was unknown. All other male and female rats survived to the end of the study.

Seizures were recorded for one 1,000 mg/kg/day male, one 250 mg/kg/day female, two 500 mg/kg/day females, and one 1,000 mg/kg/day female; seizures were reported only once for each animal. For all the female rats, the seizures were noted prior to blood collection on day 29, and for the one male, the seizure was noted on day 22; the male animal recovered and then died on day 24. The cause of the seizures was unknown, and they were not repetitive. Additionally, in 1,000 mg/kg/day females, one rat was lethargic, two had ruffled fur, and two were thin; these findings were considered related to treatment.

The mean body weights of 62.6, 125, 250, and 500 mg/kg/day males were within 10% of the vehicle controls throughout the study (Table 4 and Figure 1). There were 17% and 19% reductions in the mean body weights of the surviving 1,000 mg/kg/day males at 15 and 22 days, respectively. The mean body weights of 62.6, 125, 250, and 500 mg/kg/day females were within 10% of the vehicle controls throughout the study. The mean body weight of females in the 1,000 mg/kg/day group was 14% less than that of the vehicle controls at the end of the study.

Table 4. Mean Body Weights and Survival of Rats in the 28-day Gavage Study of Perfluorobutane Sulfonic Acid[a]

Day	Vehicle Control		62.6 mg/kg/day			125 mg/kg/day			250 mg/kg/day			500 mg/kg/day			1,000 mg/kg/day		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
Male																	
1	263	10	264	100	10	265	101	10	267	102	10	266	101	10	266	101	10
8	285	10	282	99	10	290	102	10	291	102	10	284	100	10	284	100	10
15	306	10	301	98	10	310	102	10	312	102	10	296	97	10	296	97	10
22	326	10	318	98	10	332	102	10	331	102	10	311	95	10	311	95	10
29	342	10	335	98	10	349	102	10	348	102	10	317	93	10	317	93	10
Female																	
1	199	10	199	100	10	199	100	10	202	101	10	199	100	10	199	100	10
8	210	10	210	102	10	204	98	10	205	99	10	208	100	10	208	100	10
15	218	10	218	101	10	216	100	10	212	98	10	207	96	10	207	96	10
22	226	10	226	100	10	222	99	10	219	97	10	209	93	9	209	93	9
29	230	10	230	100	10	224	97	10	224	97	9	213	92	9	213	92	9

[a]One-half the dose was administered twice daily.

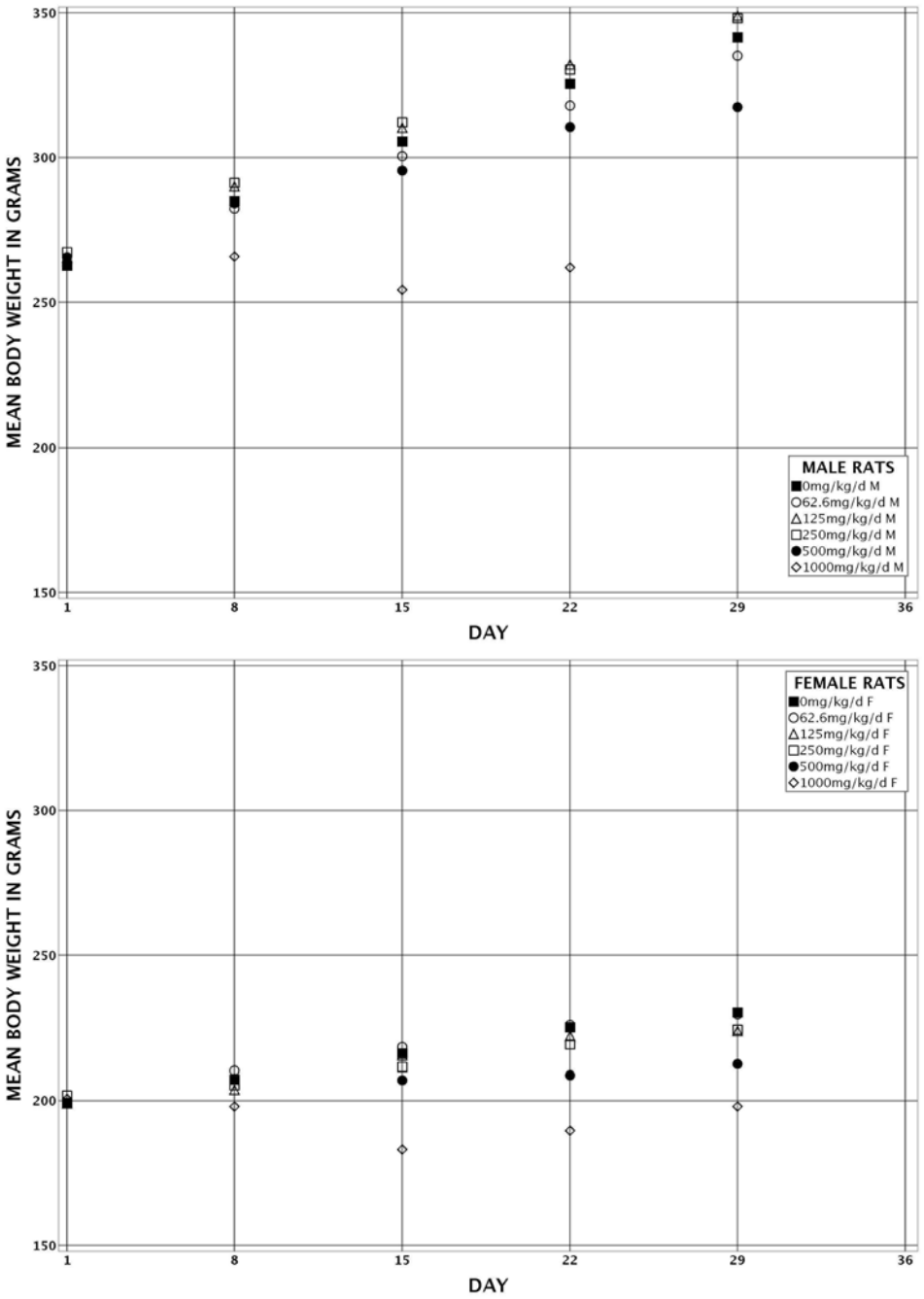


Figure 1. Growth Curves for Rats Administered Perfluorobutane Sulfonic Acid by Gavage for 28 Days

Parent compound concentrations are reported in ng/mL and μM for comparison to other PFAS tested (Table 5). Plasma concentrations of PFBS increased with increasing dose in both males and females. Males generally had higher (5- to 18-fold) plasma concentrations compared to females across all dose groups. Plasma concentrations normalized to dose administered (μM/mmol/kg/day) increased with increasing dose. In females, the normalized plasma concentration was consistent between 62.6 and 250 mg/kg/day, but was more than fourfold higher in the 500 and 1,000 mg/kg/day groups compared to the lower doses (note that the 1,000 mg/kg/day group is based on sampling of two animals). There were quantifiable levels of PFBS in male controls that were 25 times lower than the lowest dose. The source of this exposure is not clear and may be a result of contamination from background exposure.

Liver concentrations were measured only in males and data are presented in ng/g and μM for comparison to other PFAS tested (conversion assumed 1 g/mL for weight to volume) (Table 5). Concentrations increased with dose and were consistent between 62.6 and 250 mg/kg/day when normalized to dose administered. However, the normalized liver concentration was higher (about 30 versus about 20 μM/mmol/kg/day) at 500 mg/kg/day compared to the lower doses. The liver/plasma ratio in males was less than one across the doses.

Compared to vehicle controls, there were dose-related and significant increases in the relative liver weight in 62.6 mg/kg/day males and absolute and relative liver weights in the 125, 250, and 500 mg/kg/day males (Table 6). In females, there were dose-related and significant increases in the relative liver weight in the 125 mg/kg/day group and absolute and relative liver weights in the 250, 500, and 1,000 mg/kg/day groups. The organ weight changes in liver appeared to correlate with histopathologic changes observed in the liver.

Table 5. Perfluorobutane Sulfonic Acid Concentrations in the Plasma and Liver of Rats in the 28-day Gavage Study of Perfluorobutane Sulfonic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
Molar Dose (mmol/kg/day)	0	0.209	0.417	0.833	1.666	3.332
Male						
n	9	10	10	10	10	0
Plasma						
Plasma Concentration (ng/mL)	90.3 ± 16.5	2,222.0 ± 476.8**	5,366.0 ± 1,041.9**	12,430.0 ± 907.8**	43,160.0 ± 6,912.1**	–
Plasma Concentration (µM)	0.3 ± 0.1	7.4 ± 1.6**	17.9 ± 3.5**	41.4 ± 3.0**	143.8 ± 23.0**	–
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	35.5 ± 7.6	42.9 ± 8.3	49.7 ± 3.6	86.3 ± 13.8	–
Liver						
Liver Concentration (ng/g)	BD	1,245.4 ± 216.9	2,436.5 ± 527.7	4,461.0 ± 400.4	15,381.0 ± 2,590.0	–
Liver Concentration (µM)[b]	BD	4.1 ± 0.7	8.1 ± 1.8	14.9 ± 1.3	51.3 ± 8.6	–
Normalized Liver Concentration (µM/mmol/kg/day)	NA	19.9 ± 3.5	19.5 ± 4.2	17.8 ± 1.6	30.8 ± 5.2	–
Liver/Plasma Ratio	BD	0.59 ± 0.05	0.44 ± 0.02	0.36 ± 0.01	0.35 ± 0.01	–
Female						
n	10	10	10	8	9	2
Plasma						
Plasma Concentration (ng/mL)	BD	154.3 ± 48.5	308.9 ± 90.1	931.0 ± 207.4	8,171.0 ± 3,385.2	25,455.0 ± 23,145.0
Plasma Concentration (µM)	BD	0.5 ± 0.2	1.0 ± 0.3	3.1 ± 0.7	27.2 ± 11.3	84.8 ± 77.1
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	2.5 ± 0.8	2.5 ± 0.7	3.7 ± 0.8	16.3 ± 6.8	25.5 ± 23.1

**Significantly different ($p \leq 0.01$) from the vehicle control group by Shirley's test.
BD = below detection; group did not have over 20% of its values above the limit of quantification and no statistical analyses were conducted for the endpoint. One-half the dose was administered twice daily. NA = not applicable; normalized value could not be calculated when the dose value was 0.
[a]Tissue concentration data are presented as mean ± standard error. Tissue concentrations were not measured in 1,000 mg/kg/day males due to 100% mortality. If over 20% of the values in a group were above the limit of quantification (plasma LOQ = 25 ng/mL; liver LOQ = 500 ng/g), then values that were below the limit of quantification were substituted with half the limit of quantification value.
[b]Density assumed to be 1.0.

Table 6. Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 28-day Gavage Study of Perfluorobutane Sulfonic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day
Male					
n	10	10	10	10	10
Necropsy Body Wt.	342 ± 7	335 ± 8	349 ± 9	348 ± 9	317 ± 6
Heart					
Absolute	1.22 ± 0.03	1.15 ± 0.04	1.19 ± 0.03	1.20 ± 0.03	1.07 ± 0.02**
Relative	3.56 ± 0.04	3.42 ± 0.06	3.41 ± 0.06	3.46 ± 0.03	3.36 ± 0.06*
R. Kidney					
Absolute	1.00 ± 0.04	0.98 ± 0.03	1.05 ± 0.03	1.07 ± 0.03	1.11 ± 0.03*
Relative	2.93 ± 0.06	2.93 ± 0.05	3.01 ± 0.07	3.07 ± 0.04	3.49 ± 0.08**
Liver					
Absolute	12.06 ± 0.46	13.40 ± 0.42	15.30 ± 0.53**	17.99 ± 0.78**	18.52 ± 0.40**
Relative	35.23 ± 0.79	39.90 ± 0.48**	43.78 ± 0.75**	51.48 ± 0.88**	58.33 ± 0.43**
Thymus					
Absolute	0.417 ± 0.026	0.394 ± 0.018	0.429 ± 0.021	0.390 ± 0.019	0.300 ± 0.015**
Relative	1.22 ± 0.07	1.17 ± 0.03	1.23 ± 0.06	1.12 ± 0.04	0.94 ± 0.04**
Female					
n	10	10	10	9	9
Necropsy Body Wt.	230 ± 4	230 ± 3	224 ± 4	224 ± 3	213 ± 4**
Heart					
Absolute	0.86 ± 0.02	0.86 ± 0.02	0.84 ± 0.02	0.80 ± 0.02	0.78 ± 0.02*
Relative	3.73 ± 0.08	3.75 ± 0.07	3.76 ± 0.07	3.56 ± 0.07	3.68 ± 0.09
R. Kidney					
Absolute	0.67 ± 0.01	0.71 ± 0.02	0.70 ± 0.01	0.71 ± 0.02	0.69 ± 0.02
Relative	2.93 ± 0.05	3.07 ± 0.04*	3.11 ± 0.04*	3.17 ± 0.08**	3.25 ± 0.05**
Liver					
Absolute	7.79 ± 0.26	8.14 ± 0.17	8.17 ± 0.20	9.39 ± 0.17**	10.49 ± 0.35**
Relative	33.83 ± 0.90	35.48 ± 0.46	36.46 ± 0.50*	41.87 ± 0.59**	49.32 ± 1.10**
Spleen					
Absolute	0.591 ± 0.028	0.586 ± 0.013	0.540 ± 0.022	0.556 ± 0.014	0.522 ± 0.012*
Relative	2.56 ± 0.10	2.56 ± 0.06	2.41 ± 0.09	2.48 ± 0.06	2.46 ± 0.03
Thymus					
Absolute	0.288 ± 0.013	0.273 ± 0.008	0.286 ± 0.015	0.274 ± 0.016	0.236 ± 0.013**
Relative	1.25 ± 0.06	1.19 ± 0.03	1.28 ± 0.07	1.23 ± 0.08	1.11 ± 0.06

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.
**$p \leq 0.01$.
[a]Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error). No data presented for 1,000 mg/kg/day males or females due to high mortality. One-half the dose was administered twice daily.

In males, absolute and relative heart and thymus weights in the 500 mg/kg/day group were significantly decreased (Table 6). Male kidney weights (absolute and relative to body weight) were increased in the 500 mg/kg/day group. In females, dose-related and significant increases in the relative right kidney weights occurred in all the dose groups. Absolute spleen, heart, and thymus weights in the 500 mg/kg/day females were significantly lower than those of the vehicle controls. The biological significance of these changes is not clear.

There was no clinical pathology interpretation for the 1,000 mg/kg/day male and female rats due to the high mortality in these groups. Mild significant decreases in the male rat erythron were observed, most consistently in those administered 125 mg/kg/day or greater; this was characterized by significantly decreased hematocrit values, hemoglobin concentrations, and erythrocyte and reticulocyte counts compared to those of the vehicle control group (Table 7). In the female rats, the reticulocyte count was significantly decreased in the 125 mg/kg/day and higher-dose groups. Several other significant changes were observed, but were not considered toxicologically relevant.

Mild significant increases in blood urea nitrogen (BUN) concentrations were observed in the 250 and 500 mg/kg/day male rats (Table 7). Mild increases in BUN without increases in creatinine is most consistent with decreased water intake (i.e., dehydration). Total protein concentrations were decreased in all male dose groups; these decreases were due to decreases in globulin concentrations, which resulted in increases in the albumin/globulin ratio. Cholesterol concentrations were decreased in all male dose groups and in the 500 mg/kg/day females, and triglyceride concentrations were decreased in the 500 mg/kg/day males.

Alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activities were significantly increased in the 500 mg/kg/day groups; occasionally, one of these enzyme activities was also increased in the 250 mg/kg/day groups (Table 7). Additionally, sorbitol dehydrogenase (SDH) activity was significantly increased in the 500 mg/kg/day male rats. Direct bilirubin concentrations in the 500 mg/kg/day males, and total and direct bilirubin concentrations in the 500 mg/kg/day females were significantly increased. Bile acid concentrations were significantly increased in the 500 mg/kg/day males and the 250 and 500 mg/kg/day females.

Total thyroxine (T4), free T4, and total triiodothyronine (T3) concentrations were significantly decreased in all male and female dose groups (Table 7). TSH levels were unchanged.

Table 7. Selected Hematology and Clinical Chemistry Data for Rats in the 28-day Gavage Study of Perfluorobutane Sulfonic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day
Male					
Hematology					
n	9	10	10	10	10
Hematocrit (%)	52.1 ± 0.7	50.5 ± 0.4*	50.3 ± 0.6*	49.9 ± 0.5**	47.9 ± 0.5**
Manual Hematocrit (%)	47 ± 1	46 ± 1	45 ± 1	45 ± 1	44 ± 1
Hemoglobin (g/dL)	15.6 ± 0.2	15.3 ± 0.1	15.1 ± 0.2*	15.0 ± 0.1*	14.4 ± 0.2**
Erythrocytes (10^6/μL)	8.80 ± 0.08	8.50 ± 0.08*	8.50 ± 0.10*	8.40 ± 0.12**	8.10 ± 0.11**
Reticulocytes (10^3/μL)	200.1 ± 6.3	179.1 ± 8.4	175.2 ± 8.1*	161.0 ± 8.4**	137.6 ± 9.5**
Clinical Chemistry					
n	9	10	10	10	9
Urea Nitrogen (mg/dL)	18.0 ± 0.6	17.8 ± 0.4	18.6 ± 0.4	20.0 ± 0.4**	20.7 ± 1.2*
Total Protein (g/dL)	6.6 ± 0.0	6.4 ± 0.1*	6.4 ± 0.1**	6.3 ± 0.1**	6.0 ± 0.1**
Albumin (g/dL)	4.3 ± 0.0	4.2 ± 0.1	4.2 ± 0.0	4.3 ± 0.0	4.3 ± 0.0
Globulin (g/dL)	2.3 ± 0.0	2.3 ± 0.1*	2.2 ± 0.0**	2.0 ± 0.1**	1.7 ± 0.0**
Albumin/Globulin Ratio	1.8 ± 0.0	1.9 ± 0.1	2.0 ± 0.0*	2.2 ± 0.1**	2.6 ± 0.1**
Cholesterol (mg/dL)	133 ± 6	110 ± 4**	93 ± 2**	75 ± 5**	33 ± 2**
Triglycerides (mg/dL)	111 ± 8	149 ± 18	121 ± 13	101 ± 15	34 ± 6**
Alanine Aminotransferase (IU/L)	60 ± 2[b]	59 ± 2	52 ± 2	65 ± 2	91 ± 7**
Alkaline Phosphatase (IU/L)	208 ± 6	207 ± 10	220 ± 9	239 ± 9*	297 ± 14**
Sorbitol Dehydrogenase (IU/L)	5 ± 1[b]	5 ± 1	5 ± 1	8 ± 1	8 ± 1*
Direct Bilirubin (mg/dL)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.04 ± 0.00**[c]
Bile Salt/Acids (μmol/L)	16.0 ± 3.4	14.0 ± 2.9	8.5 ± 1.2	17.5 ± 1.4	30.6 ± 4.5*
Total Thyroxine (μg/dL)	3.34 ± 0.18	0.90 ± 0.09**	0.22 ± 0.05**	0.10 ± 0.03**	0.29 ± 0.07*
Free Thyroxine (ng/dL)	2.09 ± 0.09	0.64 ± 0.04**	0.32 ± 0.01**	0.30 ± 0.00**	0.30 ± 0.00*
Total Triiodothyronine (ng/dL)	117.76 ± 8.31	87.85 ± 5.00*	64.48 ± 3.08**	60.20 ± 4.10**	50.44 ± 0.44**
Thyroid Stimulating Hormone (ng/mL)	23.27 ± 2.79	26.64 ± 3.93	25.51 ± 2.53	23.76 ± 2.58	32.19 ± 3.15

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day
Female					
n	10	10	10	9	9
Hematology					
Reticulocytes ($10^3/\mu L$)	208.5 ± 16.6	181.2 ± 10.5	152.1 ± 6.3**	122.4 ± 17.7**	110.9 ± 15.8**
Clinical Chemistry					
Cholesterol (mg/dL)	103 ± 4	94 ± 3	94 ± 5	105 ± 7	85 ± 4*
Alanine Aminotransferase (IU/L)	48 ± 1	46 ± 2	48 ± 3	60 ± 2**	71 ± 5**
Alkaline Phosphatase (IU/L)	147 ± 4	147 ± 8	153 ± 10	167 ± 11	195 ± 8**
Aspartate Aminotransferase (IU/L)	54 ± 1	58 ± 1	63 ± 7	58 ± 1	61 ± 2**
Total Bilirubin (mg/dL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0**
Direct Bilirubin (mg/dL)	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.04 ± 0.00**
Bile Salt/Acids (μmol/L)	9.8 ± 2.3	7.6 ± 1.0	14.9 ± 3.3	26.2 ± 5.4**	52.4 ± 6.7**
Total Thyroxine (μg/dL)	3.10 ± 0.15	1.48 ± 0.09**	1.12 ± 0.05**	0.90 ± 0.08**	0.97 ± 0.09*
Free Thyroxine (ng/dL)	1.54 ± 0.08	0.72 ± 0.05**	0.55 ± 0.03**	0.48 ± 0.04**	0.36 ± 0.03*
Total Triiodothyronine (ng/dL)	89.29 ± 5.57	61.81 ± 3.34**	61.53 ± 3.33**	52.37 ± 1.87**	51.28 ± 0.87*
Thyroid Stimulating Hormone (ng/mL)	11.92 ± 1.29	14.56 ± 1.06	12.55 ± 1.06	14.40 ± 1.15	13.76 ± 0.99

*Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. No data presented for 1,000 mg/kg/day males or females due to high mortality. Statistical tests were performed on unrounded data. One-half the dose was administered twice daily.
[b]n = 8.
[c]n = 10.

Male and female rats administered PFBS exhibited a significant increase in expression of *Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2* compared to the controls, indicating significant increased peroxisome proliferator-activated receptor alpha (PPARα) and CAR activity (Table 8). Males displayed a greater fold increase in PPARα-related gene expression (*Cyp4a1)* compared to controls than females when compared to their controls, whereas increases in expression of CAR-related genes (*Cyp2b1, Cyp2b2*) were more prominent in female rats. Expression of *Acox1* was the least sensitive of the genes evaluated, whereas *Cyp2b1* displayed the greatest fold changes in both males and females when compared to their respective controls. Acyl-CoA oxidase activity was increased in the 250 and 500 mg/kg/day groups of males compared to controls.

Cauda epididymal sperm counts (both total and normalized to weight) were generally lower (11% to 13%) in assessed males administered PFBS than in the vehicle controls; however, none of these changes attained statistical significance (Table A-1). The testicular spermatid count in 250 mg/kg/day males was lower (10%) than the vehicle control group. When normalized to total testicular weight, counts in the 250 and 500 mg/kg/day groups were 12% and 10% lower, respectively, than the vehicle control group. These differences did not attain statistical significance, but the trend was significant. Left testis and left epididymis weights were not affected by PFBS administration. The histopathologic finding of germinal epithelium degeneration in the testis was noted in one male in the 1,000 mg/kg/day group (sperm assessments were not made in this group due to early mortality). Serum testosterone levels assessed at necropsy in dosed males were similar to the vehicle control group level (Table A-1).

Inspection of the daily vaginal cytology data indicated that the 250 and 500 mg/kg/day females displayed abnormal cyclicity with respect to diestrus (which comprised 68% and 94% of the daily observations for these groups versus 55% in the vehicle control) (Table 9 and Table A-2, Figure A-1). Markov analysis demonstrated that females administered 250 or 500 mg/kg/day PFBS displayed alteration in the estrous cycle (extended diestrus in the 250 mg/kg/day females, irregular or not cycling in the 500 mg/kg/day females). Females in the 500 mg/kg/day dose group were not cycling (Table 9 and Figure A-1).

Table 8. Hepatic Parameters for Rats in the 28-day Gavage Study of Perfluorobutane Sulfonic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
Male						
n	10	10	10	10	10	0
Enzyme Activity						
Acyl-CoA Oxidase (nmol/min/mg)	2.048 ± 0.063	2.042 ± 0.073	2.110 ± 0.067	4.051 ± 0.114**	8.896 ± 0.575**	–
Gene Expression						
Acox1	1.04 ± 0.10	1.15 ± 0.17	1.60 ± 0.15*	2.44 ± 0.22**	5.04 ± 0.63**	–
Cyp4a1	1.12 ± 0.17	2.83 ± 0.49**	5.57 ± 0.64**	11.85 ± 1.43**	26.72 ± 3.26**	–
Cyp2b1	1.59 ± 0.41	11.66 ± 2.78**	32.78 ± 6.98**	50.37 ± 17.55**	99.69 ± 24.85**	–
Cyp2b2	1.27 ± 0.25	7.72 ± 1.42**	12.21 ± 1.03**	18.34 ± 1.83**	32.92 ± 4.06**	–
Female						
n	10	10	10	9	9	2
Gene Expression						
Acox1	1.10 ± 0.18	1.44 ± 0.15	1.84 ± 0.29	2.56 ± 0.33**	3.45 ± 0.48**	5.04 ± 0.07**
Cyp4a1	1.13 ± 0.19	1.61 ± 0.17*	1.93 ± 0.36*	2.43 ± 0.26**	5.12 ± 1.27**	8.83 ± 1.71**
Cyp2b1	1.51 ± 0.42	48.97 ± 12.36**	152.85 ± 27.72**	392.32 ± 125.3**	770.07 ± 189.3**	2,102.7 ± 1,712**
Cyp2b2	1.89 ± 0.75	36.89 ± 6.65**	81.44 ± 15.88**	131.73 ± 19.14**	220.07 ± 40.68**	265.25 ± 1.42**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Hepatic parameters were not measured in 1,000 mg/kg/day males due to 100% mortality. Gene expression data are presented as fold change values from the base-signal values. One-half the dose was administered twice daily.

Table 9. Estrous Cycle Characterization for Female Rats in the 28-day Gavage Study of Perfluorobutane Sulfonic Acid[a]

	Vehicle Control	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day
n	10	10	5	0
Necropsy Body Wt. (g)	230 ± 4	224 ± 4	224 ± 3[b]	213 ± 4**[b]
Number of Estrous Cycles	2.0 ± 0.21	2.0 ± 0.21	1.2 ± 0.20	–[c]
Estrous Cycle Length (days)	5.0 ± 0.20	5.0 ± 0.09	5.1 ± 0.10	–
Estrous Stages (% of cycle)				
Diestrus	55.0	55.1	67.8	93.6
Proestrus	8.8	5.1	5.9	0.9
Estrus	33.8	38.5	21.7	3.6
Metestrus	1.9	1.3	1.3	1.8
Uncertain Diagnosis	0.6	0.0	3.3	0.0
Markov Analysis				
	Overall	**125 mg/kg/day**	**250 mg/kg/day**	**500 mg/kg/day**
Overall Tests	$p < 0.001$	$p = 0.449$	$p < 0.001$	$p < 0.001$
Extended Diestrus	$p < 0.001$	$p = 0.063$	$p < 0.001$	$p < 0.001$

**Significantly different ($p \leq 0.01$) from the vehicle control group by Williams' or Dunnett's test.
[a]Necropsy body weights, number of estrous cycles, and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunn's test (estrous cycle number and length). N indicates that the dose group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dose group had more departures from normal cycling than did the vehicle control group. Tests for equality of all transition matrices are shown in the vehicle control column (Markov analysis). One-half the dose was administered twice daily.
[b]n = 9.
[c]Animals were not cycling.

Pathology

The morphologic features of the lesions discussed in this section are presented in the Histopathologic Descriptions section following the Wyeth-14,643 results.

Liver: Compared to the respective vehicle control groups, the incidences of hepatocyte hypertrophy were significantly increased in 125 mg/kg/day and greater groups of males and in 500 and 1,000 mg/kg/day groups of females (Table 10). Hepatocyte cytoplasmic alteration was significantly increased in 500 and 1,000 mg/kg/day males and females. The average severity of these changes increased with increasing dose. In general, both lesions were of minimal to mild severity. Hepatocyte necrosis was increased, but not significantly, in 1,000 mg/kg/day females, and the necrosis was of minimal severity. One male in the 1,000 mg/kg/day group had hepatocyte necrosis.

Bone Marrow: The incidence of bone marrow hypocellularity was significantly increased in the 1,000 mg/kg/day males and females (Table 10). Bone marrow hypocellularity was mild to marked in severity.

Nose: In males, the incidences of olfactory epithelium degeneration and olfactory epithelium hyperplasia in the 250, 500, and 1,000 mg/kg/day groups and olfactory epithelium inflammation suppurative and olfactory epithelium necrosis in the 1,000 mg/kg/day groups were significantly increased compared to those of the vehicle controls (Table 10). The average severity of these changes, except olfactory epithelium hyperplasia, increased with increasing dose. In females, the incidences of olfactory epithelium degeneration at 125, 250, 500, and 1,000 mg/kg/day, olfactory epithelium hyperplasia at 250, 500, and 1,000 mg/kg/day, and olfactory epithelium inflammation suppurative and olfactory epithelium necrosis at 1,000 mg/kg/day were significantly increased compared to vehicle controls (Table 10). The average severity of these changes increased with increasing dose.

Stomach, Forestomach: In males, the incidence of epithelium hyperplasia was significantly increased in the 1,000 mg/kg/day group (Table 10). Epithelium hyperplasia was of minimal to mild severity.

Thymus: The incidences of atrophy were significantly increased in the 1,000 mg/kg/day males and females (Table 10). Severity of atrophy was mild to marked.

Kidney: The incidence of papilla necrosis was significantly increased in 1,000 mg/kg/day females (Table 10). One male rat in the 1,000 mg/kg/day group also had papilla necrosis (Table 10). The change was of minimal to mild severity.

Table 10. Incidences of Selected Nonneoplastic Lesions in Rats in the 28-day Gavage Study of Perfluorobutane Sulfonic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
Male						
Liver[b]	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration[c]	0	0	1 (1.0)[d]	0	10** (1.0)	9** (1.6)
Hepatocyte, Hypertrophy	0	0	9** (1.0)	10** (1.3)	10** (1.7)	9** (2.1)
Necrosis	0	0	0	0	0	1 (3.0)
Bone Marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hypocellularity	0	0	0	0	0	10** (2.6)
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory Epithelium, Degeneration	0	0	3 (1.0)	7** (1.4)	9** (1.6)	9** (2.3)
Olfactory Epithelium, Hyperplasia	0	0	2 (1.0)	5* (1.2)	9** (1.6)	5* (1.4)
Olfactory Epithelium, Inflammation, Suppurative	0	0	0	2 (1.0)	2 (1.5)	9** (2.4)
Olfactory Epithelium, Necrosis	0	0	0	0	0	7** (2.0)
Stomach, Forestomach	(10)	(0)	(0)	(10)	(10)	(10)
Epithelium, Hyperplasia	0	–	–	0	1 (1.0)	6** (1.2)
Thymus	(10)	(10)	(10)	(10)	(10)	(10)
Atrophy	0	0	0	0	0	5* (3.2)
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Papilla, Necrosis	0	0	0	0	0	1 (2.0)

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day	1,000 mg/kg/day
Female						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration	0	0	0	0	9** (1.0)	10** (2.2)
Hepatocyte, Hypertrophy	0	0	0	0	8** (1.0)	10** (2.1)
Necrosis	0	0	0	0	0	3 (1.0)
Bone Marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hypocellularity	0	0	0	0	0	9** (2.9)
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory Epithelium, Degeneration	0	0	4* (1.0)	9** (1.3)	9** (1.9)	7** (2.0)
Olfactory Epithelium, Hyperplasia	0	0	0	8** (1.1)	9** (1.4)	4* (1.5)
Olfactory Epithelium, Inflammation, Suppurative	0	0	1 (1.0)	1 (1.0)	2 (1.5)	8** (2.5)
Olfactory Epithelium, Necrosis	0	0	0	0	0	6** (1.8)
Thymus	(10)	(10)	(10)	(10)	(10)	(10)
Atrophy	0	0	0	0	0	5* (3.0)
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Papilla, Necrosis	0	0	0	0	0	4* (1.5)

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test.
**$p \leq 0.01$.
[a]One-half the dose was administered twice daily. High mortality observed in the 1,000 mg/kg/day groups (survival at terminal necropsy: 0/10 males, 2/10 females).
[b]Number of animals with tissue examined microscopically.
[c]Number of animals with lesion.
[d]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Perfluorohexane Sulfonate Potassium Salt (PFHxSK)

All rats survived to scheduled euthanasia (Table 11). There were no significant treatment-related clinical observations in male or female rats.

The mean body weights of dose groups of males and females were within 10% of the respective vehicle control groups throughout the study (Table 11 and Figure 2).

Parent chemical concentrations are reported in ng/mL and μM for comparison to other PFAS tested (Table 12). Plasma concentrations of PFHxS increased with increasing dose in males and females. Although females were administered doses five times higher than those administered to males, the female plasma concentrations were about half of male concentrations. When adjusted for dose (μM/mmol/kg/day), plasma concentrations were 9- to 10-fold higher in males compared to females. In both males and females, normalized plasma concentrations steadily decreased with increasing dose. The quantifiable levels of PFHxS in male and female vehicle controls were 556 and 231 times lower than the lowest doses, respectively. The source of this exposure is not clear and may be a result of contamination from background exposure.

Liver concentrations were measured only in males and data are presented in ng/g and μM for comparison to other PFAS tested (conversion assumed 1 g/mL for weight to volume) (Table 12). Concentrations increased with dose, but when normalized to dose, liver concentrations steadily decreased as dose increased similar to the normalized plasma concentrations. The liver/plasma ratio ranged from 0.61 to 1.22.

In males, there were dose-related and significant increases in the absolute and relative liver weights in the 1.25 mg/kg/day and greater groups (Table 13). In females, there were dose-related and significant increases in the absolute and relative liver weights in all dose groups. Liver weights appeared to correlate with histopathologic changes in the liver.

In males, the relative right adrenal gland weight in the 2.5 mg/kg/day group and absolute and relative weights in the 5 and 10 mg/kg/day groups were significantly lower than those of the vehicle control group (Table 13). Relative right kidney weight in the 10 mg/kg/day males was significantly increased. In females, absolute right adrenal gland weights in the 12.5, 25, and 50 mg/kg/day groups and the relative right adrenal gland weight in the 50 mg/kg/day group were significantly increased. Biological significance of these changes is not clear.

Reticulocyte counts were significantly decreased in the 1.25 mg/kg/day and higher male groups (Table 14).

The globulin concentration was significantly decreased in the 10 mg/kg/day male group; this decrease resulted in an increase in the albumin:globulin ratio in this group (Table 14). Cholesterol concentrations were significantly decreased in the 1.25 mg/kg/day and higher males, whereas triglycerides were decreased in the 2.5 mg/kg and higher males.

Free T4, total T4, and total T3 concentrations were significantly decreased in all dose groups of male rats (Table 14). In female rats, significant decreases were seen in total T4 at 6.25 mg/kg/day and greater and free T4 at 12.5 mg/kg/day and greater.

Male rats administered PFHxSK exhibited significant increases in expression of *Acox1, Cyp4a1, Cyp2b1,* and *Cyp2b2* compared to controls, indicating significantly increased PPARα and CAR

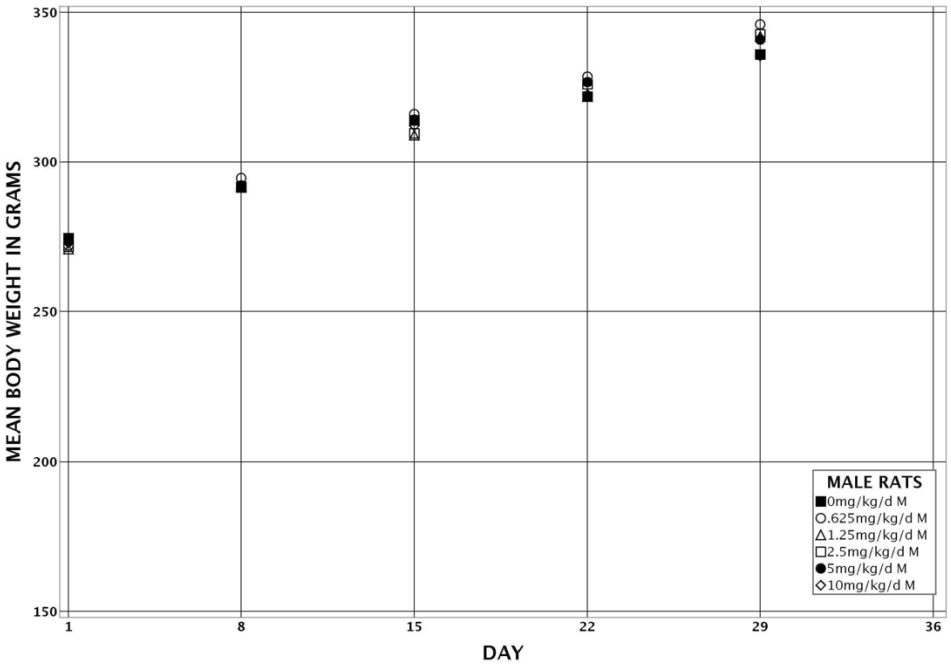
activity (Table 15). *Acox1* expression was the least sensitive of the genes evaluated. Female rats displayed increases in *Cyp2b1* and *Cyp2b2* expression compared to the control only without any significant increase in expression of PPARα-related genes (*Acox1* and *Cyp4a1*) compared to controls. *Cyp2b1* expression displayed the greatest fold changes in both males and females compared to the respective controls. Acyl-CoA oxidase activity was increased in the male liver in the 5 and 10 mg/kg/day groups compared to the controls.

Assessed male rats did not display any changes in sperm counts or motility (Table A-3). Left testis and epididymis weights in rats administered PFHxSK were similar to the vehicle control group. No treatment-related testicular or epididymal lesions were noted and the serum testosterone levels assessed at necropsy were similar to the vehicle control group (Table A-3).

Inspection of the daily vaginal cytology data indicated that the females administered PFHxSK were cycling normally (Table A-4 and Table A-5; Figure A-2).

Table 11. Mean Body Weights and Survival of Rats in the 28-day Gavage Study of Perfluorohexane Sulfonate Potassium Salt

	Vehicle Control		0.625 mg/kg/day			1.25 mg/kg/day			2.5 mg/kg/day			5 mg/kg/day			10 mg/kg/day		
Day	**Av. Wt. (g)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**
Male																	
1	275	10	272	99	10	271	99	10	272	99	10	273	100	10	273	100	10
8	292	10	295	101	10	292	100	10	292	100	10	292	100	10	291	100	10
15	314	10	316	101	10	309	99	10	310	99	10	314	100	10	312	99	10
22	322	10	329	102	10	322	100	10	326	101	10	327	102	10	323	100	10
29	336	10	346	103	10	342	102	10	343	102	10	341	102	10	335	100	10
	Vehicle Control		**3.12 mg/kg/day**			**6.25 mg/kg/day**			**12.5 mg/kg/day**			**25 mg/kg/day**			**500 mg/kg/day**		
	Av. Wt. (g)	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**
Female																	
1	201	10	200	100	10	202	100	10	199	99	10	198	99	10	200	99	10
8	205	10	208	101	10	206	101	10	210	102	10	206	100	10	208	101	10
15	213	10	219	103	10	217	102	10	219	103	10	216	101	10	216	101	10
22	217	10	224	103	10	229	105	10	225	104	10	223	103	10	222	102	10
29	224	10	233	104	10	236	106	10	227	102	10	230	103	10	230	103	10



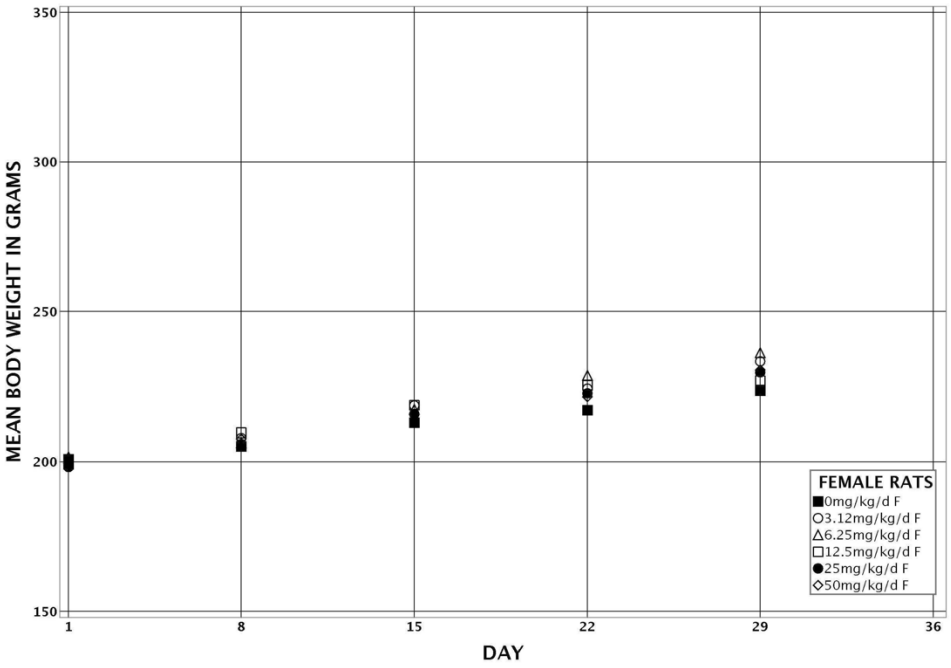


Figure 2. Growth Curves for Rats Administered Perfluorohexane Sulfonate Potassium Salt by Gavage for 28 Days

Table 12. Perfluorohexane Sulfonic Acid Concentrations in the Plasma and Liver of Rats in the 28-day Gavage Study of Perfluorohexane Sulfonate Potassium Salt[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
n	10	10	10	10	10	10
Molar Dose (mmol/kg/day)	0	0.0016	0.0031	0.0062	0.0125	0.025
Male						
Plasma						
Plasma Concentration (ng/mL)	102.2 ± 14.4	66,760.0 ± 3,518.3**	92,080.0 ± 3,347.9**	129,000.0 ± 5,503.5**	161,700.0 ± 2,512.4**	198,300.0 ± 4,955.7**
Plasma Concentration (μM)	0.3 ± 0.0	166.9 ± 8.8**	230.1 ± 8.4**	322.4 ± 13.8**	404.1 ± 6.3**	495.6 ± 12.5**
Normalized Plasma Concentration (μM/mmol/kg/day)	NA	106,816.0 ± 5,629.3	73,664.0 ± 2,678.3	51,600.0 ± 2,201.4	32,340.0 ± 502.5	19,830.0 ± 499.6
Liver						
Liver Concentration (ng/g)	BD	39,880.0 ± 1,314.2	58,590.0 ± 1,975.8	98,250.0 ± 3,072.9**	161,700.0 ± 9,669.0	241,300.0 ± 9,089.7
Liver Concentration (μM)b	BD	99.7 ± 3.3	146.4 ± 4.9	245.6 ± 7.7	404.1 ± 24.2	603.1 ± 22.7
Normalized Liver Concentration (μM/mmol/kg/day)	NA	63,808.0 ± 2,102.7	46,872.0 ± 1,580.6	39,300.0 ± 1,229.1	32,340.0 ± 1,933.8	24,130.0 ± 909.0
Liver/Plasma Ratio	BD	0.61 ± 0.03	0.64 ± 0.02	0.77 ± 0.04	1.00 ± 0.05	1.22 ± 0.04
	Vehicle Control	**3.12 mg/kg/day**	**6.25 mg/kg/day**	**12.5 mg/kg/day**	**25 mg/kg/day**	**50 mg/kg/day**
Molar Dose (mmol/kg/day)	0	0.0078	0.0156	0.0312	0.0625	0.125
Female						
Plasma						
Plasma Concentration (ng/mL)	174.4 ± 22.3	37,030.0 ± 1,650.9**	50,410.0 ± 1,552.2**	63,820.0 ± 3,201.5**	820.0 ± 3,739.5**	95,510.0 ± 3,745.5**
Plasma Concentration (μM)	0.4 ± 0.1	92.5 ± 4.1**	126.0 ± 3.9**	159.5 ± 8.0**	209.5 ± 9.3**	238.7 ± 9.4**
Normalized Plasma Concentration (μM/mmol/kg/day)	NA	11,868.6 ± 529.1	8,065.6 ± 248.3	5,105.6 ± 256.1	3,352.8 ± 149.6	1,910.2 ± 74.9

**Significantly different ($p \leq 0.01$) from the vehicle control group by Shirley's test.
BD = below detection; group did not have over 20% of its values above the limit of quantification and no statistical analyses were conducted for the endpoint.
[a]Tissue concentration data are presented as mean ± standard error. If over 20% of the values in a group were above the limit of quantification (plasma LOQ = 23.4 ng/mL; liver LOQ = 500 ng/g), then values that were below the limit of quantification were substituted with half the limit of quantification value. NA = not applicable; normalized value could not be calculated when the dose value was 0.
[b]Density assumed to be 1.0.

Table 13. Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 28-day Gavage Study of Perfluorohexane Sulfonate Potassium Salt[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
n	10	10	10	10	10	10
Male						
Necropsy Body Wt.	336 ± 7	346 ± 8	342 ± 7	343 ± 6	341 ± 5	335 ± 6
R. Adrenal gland						
Absolute	0.0212 ± 0.0006	0.0211 ± 0.0008	0.0212 ± 0.0008	0.0192 ± 0.0006	0.0185 ± 0.0005*	0.0196 ± 0.0007*
Relative	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00*	0.05 ± 0.00*	0.06 ± 0.00*
R. Kidney						
Absolute	1.01 ± 0.03	0.99 ± 0.03	1.01 ± 0.02	1.01 ± 0.02	1.03 ± 0.03	1.08 ± 0.03
Relative	3.01 ± 0.07	2.85 ± 0.07	2.97 ± 0.04	2.94 ± 0.05	3.02 ± 0.08	3.23 ± 0.05*
Liver						
Absolute	11.36 ± 0.32	12.10 ± 0.36	12.58 ± 0.25*	13.30 ± 0.35**	15.24 ± 0.56**	17.43 ± 0.52**
Relative	33.77 ± 0.36	34.91 ± 0.43	36.81 ± 0.39**	38.79 ± 0.62**	44.61 ± 1.16**	51.96 ± 1.24**
	Vehicle Control	**3.12 mg/kg/day**	**6.25 mg/kg/day**	**12.5 mg/kg/day**	**25 mg/kg/day**	**50 mg/kg/day**
Female						
Necropsy Body Wt.	224 ± 3	233 ± 4	236 ± 5*	227 ± 4	230 ± 3	230 ± 3
R. Adrenal gland						
Absolute	0.0237 ± 0.0008	0.0247 ± 0.0015	0.0248 ± 0.0013	0.0273 ± 0.0004*	0.0267 ± 0.0011*	0.0283 ± 0.0011**
Relative	0.11 ± 0.00	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.01*
Liver						
Absolute	7.14 ± 0.20	8.02 ± 0.26*	8.24 ± 0.30*	7.86 ± 0.25*	8.07 ± 0.16**	8.80 ± 0.24**
Relative	31.92 ± 0.68	34.36 ± 0.88*	34.80 ± 0.72**	34.58 ± 0.66**	35.14 ± 0.51**	38.16 ± 0.75**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.
**$p \leq 0.01$.
[a]Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

Table 14. Selected Hematology and Clinical Chemistry Data for Rats in the 28-day Gavage Study of Perfluorohexane Sulfonate Potassium Salt[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
n	10	10	10	10	10	10
Male						
Hematology						
Reticulocytes (10^3/μL)	182.0 ± 7.4	163.6 ± 9.1	155.4 ± 7.6*	152.6 ± 5.7*	133.7 ± 7.4**	141.0 ± 6.6**
Clinical Chemistry						
Total Protein (g/dL)	6.4 ± 0.1	6.2 ± 0.0	6.3 ± 0.1	6.2 ± 0.1	6.4 ± 0.1	6.3 ± 0.1
Albumin (g/dL)	4.2 ± 0.1	4.0 ± 0.0	4.1 ± 0.0	4.1 ± 0.1	4.2 ± 0.1	4.4 ± 0.1
Globulin (g/dL)	2.2 ± 0.0	2.2 ± 0.0	2.3 ± 0.1	2.1 ± 0.0	2.1 ± 0.0	1.9 ± 0.1**
Albumin/Globulin Ratio	1.9 ± 0.0	1.9 ± 0.0	1.8 ± 0.1	1.9 ± 0.1	2.0 ± 0.0	2.3 ± 0.1**
Cholesterol (mg/dL)	109 ± 4	101 ± 3	96 ± 3*	92 ± 3**	87 ± 5**	73 ± 3**
Triglycerides (mg/dL)	138 ± 6	124 ± 7	136 ± 13	107 ± 14*	89 ± 8**	87 ± 11**
Testosterone (ng/mL)	4.60 ± 0.68	7.74 ± 1.44	6.50 ± 1.66	8.11 ± 1.75	5.88 ± 1.14	7.14 ± 1.27
Total Thyroxine (μg/dL)	4.24 ± 0.23	2.39 ± 0.08**	1.70 ± 0.06**	1.47 ± 0.07**	1.54 ± 0.09**	1.66 ± 0.05**
Free Thyroxine (ng/dL)	1.74 ± 0.10	0.82 ± 0.07**	0.48 ± 0.03**	0.36 ± 0.02**	0.39 ± 0.03**	0.39 ± 0.03**
Total Triiodothyronine (ng/dL)	85.18 ± 5.74	66.21 ± 4.20*	58.67 ± 2.87**	54.25 ± 2.31**	52.50 ± 1.42**	56.83 ± 3.96**
Thyroid Stimulating Hormone (ng/mL)	17.31 ± 2.39	18.58 ± 1.95	20.78 ± 1.82	21.88 ± 3.13	19.69 ± 1.75	24.96 ± 4.31
	Vehicle Control	**3.12 mg/kg/day**	**6.25 mg/kg/day**	**12.5 mg/kg/day**	**25 mg/kg/day**	**50 mg/kg/day**
Female						
Clinical Chemistry						
Total Thyroxine (μg/dL)	3.99 ± 0.19	3.53 ± 0.20	3.37 ± 0.17*	2.97 ± 0.11**	2.96 ± 0.19**	2.69 ± 0.15**
Free Thyroxine (ng/dL)	1.52 ± 0.10	1.32 ± 0.10	1.28 ± 0.13	1.01 ± 0.05**	1.07 ± 0.09**	0.94 ± 0.08**
Total Triiodothyronine (ng/dL)	111.83 ± 7.60	98.89 ± 4.94	99.05 ± 7.29	96.71 ± 6.36	96.89 ± 6.04	91.51 ± 5.49
Thyroid Stimulating Hormone (ng/mL)	12.40 ± 0.89	15.66 ± 1.07	15.72 ± 1.64	17.46 ± 1.94	14.02 ± 1.04	14.50 ± 1.13

*Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.

Table 15. Hepatic Parameters for Rats in the 28-day Gavage Study of Perfluorohexane Sulfonate Potassium Salt[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
Male						
n	10	10	10	10	10	10
Enzyme Activity						
Acyl-CoA Oxidase (nmol/min/mg)	2.014 ± 0.039	2.1612 ± 0.062	1.906 ± 0.029	2.077 ± 0.062	2.783 ± 0.247**	4.579 ± 0.173**
Gene Expression						
Acox1	1.03 ± 0.10	0.86 ± 0.08	1.00 ± 0.07	1.35 ± 0.11	1.90 ± 0.23**	2.88 ± 0.20**
Cyp4a1	1.08 ± 0.14	1.32 ± 0.12	2.29 ± 0.26**	5.62 ± 0.64**	9.68 ± 1.32**	17.10 ± 1.53**
Cyp2b1	1.10 ± 0.15	1.58 ± 0.31	3.97 ± 1.08**	8.397 ± 2.15**	22.08 ± 5.95**	29.61 ± 8.67**
Cyp2b2	1.28 ± 0.34	1.75 ± 0.22	4.22 ± 0.51**	5.77 ± 0.69**	9.12 ± 1.29**	13.35 ± 1.00**
	Vehicle Control	**3.12 mg/kg/day**	**6.25 mg/kg/day**	**12.5 mg/kg/day**	**25 mg/kg/day**	**50 mg/kg/day**
Female						
n	9	10	10	10	10	10
Gene Expression						
Acox1	0.77 ± 0.06	0.74 ± 0.05	0.85 ± 0.29	0.58 ± 0.04	0.69 ± 0.04	0.77 ± 0.04
Cyp4a1	0.74 ± 0.06	0.80 ± 0.04	0.96 ± 0.36*	0.68 ± 0.05	0.79 ± 0.05	0.77 ± 0.05
Cyp2b1	1.14 ± 0.42	4.40 ± 1.22*	9.93 ± 1.67**	8.08 ± 1.54**	24.54 ± 4.40**	43.46 ± 6.67**
Cyp2b2	1.02 ± 0.41	4.26 ± 1.35*	8.12 ± 1.47**	6.78 ± 1.34**	17.95 ± 3.11**	25.99 ± 2.91**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Gene expression data are presented as fold change values from the base-signal values.

Pathology

The morphologic features of the lesions discussed in this section are presented in the Histopathologic Descriptions section following the Wyeth-14,643 results.

Liver: Compared to the incidence in vehicle controls, the incidences of hepatocyte hypertrophy were significantly increased in 2.5, 5, and 10 mg/kg/day males (Table 16). The severity of hepatocyte hypertrophy was generally mild to marked.

Nose: The incidences of olfactory epithelium degeneration and olfactory epithelium hyperplasia in the 50 mg/kg/day females were significantly increased compared to the vehicle control incidences (Table 16). There was also an increase in the incidence of olfactory epithelium inflammation suppurative in this group. These changes were primarily minimal to mild in severity.

Table 16. Incidences of Selected Nonneoplastic Lesions in Rats in the 28-day Gavage Study of Perfluorohexane Sulfonate Potassium Salt

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
Male						
Liver[a]	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Hypertrophy[b]	0	0	0	4* (2.0)[c]	9** (2.0)	10** (3.2)
	Vehicle Control	**3.12 mg/kg/day**	**6.25 mg/kg/day**	**12.5 mg/kg/day**	**25 mg/kg/day**	**50 mg/kg/day**
Female						
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory Epithelium, Degeneration	0	0	0	0	0	8** (1.1)
Olfactory Epithelium, Hyperplasia	0	0	0	0	0	5* (1.0)
Olfactory Epithelium, Inflammation, Suppurative	0	0	0	0	0	3 (1.0)

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test.
**$p \leq 0.01$.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Perfluorooctane Sulfonic Acid (PFOS)

All rats survived to scheduled euthanasia except for one female in the 5 mg/kg/day group (Table 17). There were no significant treatment-related clinical observations in male or female rats.

The mean body weights of all dose male and female groups were within 10% of those of the respective vehicle control groups throughout the study (Table 17 and Figure 3).

Parent compound concentrations are reported in ng/mL and μM for comparison to other PFAS tested (Table 18). Plasma concentrations of PFOS increased with increasing dose in males and females. Plasma concentrations in females were generally similar to males. Dose-normalized plasma concentrations (μM/mmol/kg/day) in males and females were generally similar (within 1.3-fold) across the dose groups, with the lowest concentration in the highest dose group in both sexes. There were quantifiable levels of PFOS in female controls that were 562 times lower than the lowest dose administered. The source of this exposure is not clear and may be a result of contamination from background exposure.

Liver concentrations were measured only in males and data are presented in ng/g and μM for comparison to other PFAS tested (conversion assumed 1 g/mL for weight to volume) (Table 18). Concentrations in males increased with increasing dose, but when normalized with dose, there was a steady decrease as dose increased. This corresponded with a decreasing liver/plasma ratio as dose increased.

In all treated male and female groups, compared to the vehicle controls, there were dose-related and significant increases in the absolute and relative liver weights (Table 19). Organ weight changes in the liver appear to correlate with histopathologic changes in the liver.

Table 17. Mean Body Weights and Survival of Rats in the 28-day Gavage Study of Perfluorooctane Sulfonic Acid

	Vehicle Control		0.312 mg/kg/day			0.625 mg/kg/day			1.25 mg/kg/day			2.5 mg/kg/day			5 mg/kg/day		
Day	**Av. Wt. (g)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**	**Av. Wt. (g)**	**Wt. (% of Controls)**	**No. of Survivors**
Male																	
1	267	10	267	100	10	266	100	10	267	100	10	266	100	10	266	100	10
8	288	10	289	100	10	289	100	10	285	99	10	286	99	10	283	98	10
15	309	10	310	100	10	308	100	10	303	98	10	308	100	10	296	96	10
22	326	10	325	100	10	323	99	10	319	98	10	321	99	10	307	94	10
29	337	10	340	101	10	338	100	10	331	98	10	333	99	10	309	92	10
Female																	
1	196	10	192	98	10	191	98	10	194	99	10	193	99	10	195	99	10
8	199	10	204	102	10	200	101	10	203	102	10	202	101	10	192	96	10
15	211	10	216	103	10	211	100	10	210	100	10	208	99	10	199	94	10
22	213	10	222	105	10	217	102	10	215	101	10	214	100	10	194	91	10
29	219	10	228	104	10	223	101	10	219	100	10	215	98	10	205	93	9

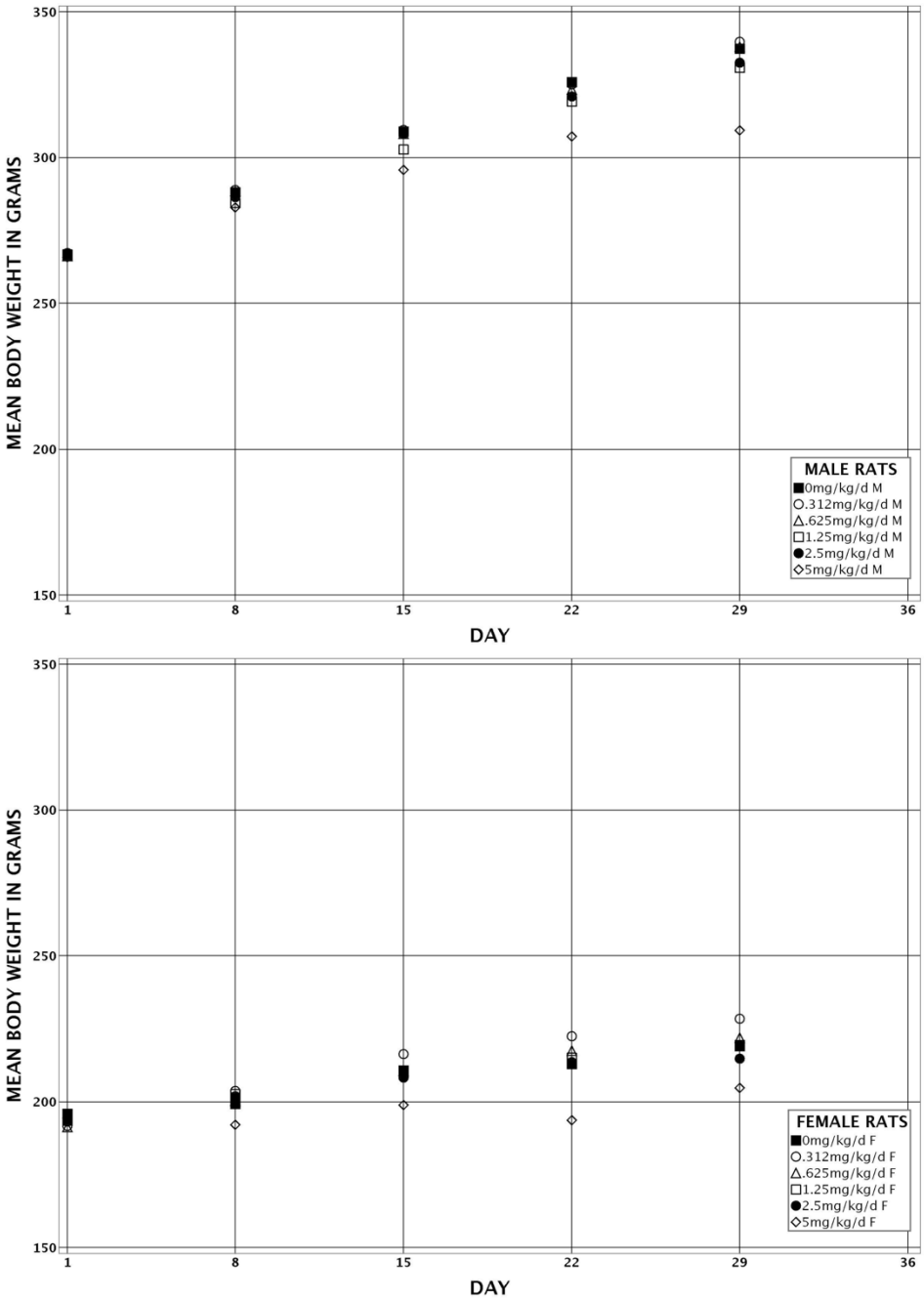


Figure 3. Growth Curves for Rats Administered Perfluorooctane Sulfonic Acid by Gavage for 28 Days

Table 18. Perfluorooctane Sulfonic Acid Concentrations in the Plasma and Liver of Rats in the 28-day Gavage Study of Perfluorooctane Sulfonic Acid[a]

	Vehicle Control	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day
Molar Dose (mmol/kg/day)	0	0.00062	0.0013	0.0025	0.005	0.01
Male						
n	10	10	10	10	10	10
Plasma						
Plasma Concentration (ng/mL)	BD	23,730.0 ± 1,113.7	51,560.0 ± 3,221.5	94,260.0 ± 3,143.8	173,700.0 ± 9,035.8	318,200.0 ± 8,867.9
Plasma Concentration (µM)	BD	47.4 ± 2.2	103..1 ± 6.4	188.5 ± 6.3	347.3 ± 18.1	636.23 ± 17.7
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	76,057.7 ± 3,569.4	82,496.0 ± 5,154.4	75,408.0 ± 2,515.1	69,480.0 ± 3,614.3	63,640.0 ± 1,773.6
Liver						
Liver Concentration (ng/g)	BD	87,170.0 ± 3,038.6	160,100.0 ± 7,208.7	286,100.0 ± 7,881.7	468,200.0 ± 12,136.1	867,100.0 ± 26,801.9
Liver Concentration (µM)[b]	BD	147.3 ± 6.1	320.1 ± 14.4	572.1 ± 15.8	936.2 ± 24.3	1,733.7 ± 53.6
Normalized Liver Concentration (µM/mmol/kg/day)	NA	279,391.0 ± 9,739.2	256,160.0 ± 11,533.9	228,880.0 ± 6,305.4	187,280.0 ± 4,854.4	173,420.0 ± 5,360.4
Liver/Plasma Ratio	BD	3.76 ± 0.24	3.29 ± 0.35	3.06 ± 0.11	2.75 ± 0.13	2.74 ± 0.08
Female						
n	10	10	10	10	10	9
Plasma						
Plasma Concentration (ng/mL)	54.3 ± 3.7	30,530.0 ± 917.6**	66,970.0 ± 1,629.5**	135,100.0 ± 3,877.1**	237,500.0 ± 5,218.0**	413,555.6 ± 8,071.1**
Plasma Concentration (µM)	0.1 ± 0.0	61.0 ± 1.8**	133.9 ± 3.3**	270.1 ± 7.8**	474.9 ± 10.4**	826.9 ± 16.1**
Normalized Plasma Concentration (µM/mmol/kg/day)	NA	97,852.6 ± 2,941.0	107,152.0 ± 2,607.1	108,080.0 ± 3,101.7	95,000.0 ± 2,087.2	82,711.1 ± 1,614.2

**Significantly different ($p \leq 0.01$) from the vehicle control group by Shirley's test.
BD = below detection; group did not have over 20% of its values above the limit of quantification and no statistical analyses were conducted for the endpoint. NA = not applicable; normalized value could not be calculated when the dose value was 0.
[a]Tissue concentration data are presented as mean ± standard error. If over 20% of the values in a group were above the limit of quantification (plasma LOQ = 25 ng/mL; liver LOQ = 500 ng/g), then values that were below the limit of quantification were substituted with half the limit of quantification value.
[b]Density assumed to be 1.0.

Table 19. Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 28-day Gavage Study of Perfluorooctane Sulfonic Acid[a]

	Vehicle Control	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day
Male						
n	10	10	10	10	10	10
Necropsy Body Wt.	337 ± 7	340 ± 5	338 ± 7	331 ± 4	333 ± 4	309 ± 4**
R. Adrenal Gland						
Absolute	0.0211 ± 0.0008	0.0180 ± 0.0010	0.0210 ± 0.0006	0.0177 ± 0.0008*	0.0190 ± 0.0012*	0.0173 ± 0.0005**
Relative	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
Liver						
Absolute	11.79 ± 0.29	13.14 ± 0.28**	14.21 ± 0.32**	15.34 ± 0.37**	17.37 ± 0.36**	18.81 ± 0.41**
Relative	34.92 ± 0.22	38.66 ± 0.47**	42.04 ± 0.48**	46.38 ± 0.99**	52.21 ± 0.93**	60.80 ± 0.77**
Spleen						
Absolute	0.688 ± 0.014	0.708 ± 0.019	0.686 ± 0.021	0.617 ± 0.025*	0.611 ± 0.033*	0.593 ± 0.019**
Relative	2.04 ± 0.05	2.09 ± 0.05	2.04 ± 0.09	1.87 ± 0.08	1.84 ± 0.10	1.92 ± 0.06
Thymus						
Absolute	0.431 ± 0.023	0.465 ± 0.013	0.412 ± 0.023	0.401 ± 0.022	0.385 ± 0.011	0.322 ± 0.018**
Relative	1.28 ± 0.08	1.37 ± 0.04	1.21 ± 0.05	1.21 ± 0.05	1.16 ± 0.03	1.04 ± 0.04**

	Vehicle Control	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day
Female						
n	10	10	10	10	10	9
Necropsy Body Wt.	219 ± 3	228 ± 4	222 ± 2	219 ± 4	215± 4	205 ± 4**
Heart						
Absolute	0.84 ± 0.01	0.87 ± 0.02	0.86 ± 0.02	0.87 ± 0.02	0.82 ± 0.02	0.72 ± 0.01**
Relative	3.84 ± 0.08	3.79 ± 0.06	3.89 ± 0.06	3.96 ± 0.07	3.80 ± 0.05	3.51 ± 0.06**
Liver						
Absolute	7.37 ± 0.18	8.26 ± 0.22*	8.20 ± 0.17*	8.59 ± 0.26**	9.17 ± 0.28**	10.92 ± 0.29**
Relative	33.56 ± 0.66	36.15 ± 0.54*	36.95 ± 0.73*	39.25 ± 1.06**	42.67 ± 0.87**	53.37 ± 1.48**
Lung						
Absolute	1.58 ± 0.07	1.78 ± 0.12	1.85 ± 0.05	2.03 ± 0.12*	1.87 ± 0.13	1.84 ± 0.12
Relative	7.23 ± 0.34	7.78 ± 0.43	8.35 ± 0.27	9.30 ± 0.55*	8.69 ± 0.54**	8.99 ± 0.58*
Thymus						
Absolute	0.312 ± 0.021	0.276 ± 0.007	0.297 ± 0.010	0.258 ± 0.014*	0.295 ± 0.013	0.251 ± 0.015*
Relative	1.42 ± 0.09	1.21 ± 0.03	1.34 ± 0.04	1.18 ± 0.06*	1.37 ± 0.05	1.23 ± 0.07

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.
**$p \leq 0.01$.
[a]Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

In males, body weights were lower in the high dose group. Absolute right adrenal gland and spleen weights in the 1.25, 2.5, and 5 mg/kg/day groups, and absolute and relative thymus weights in the 5 mg/kg/day group were significantly decreased (Table 19). In females, body weights were lower in the high dose group. Absolute and relative heart weights in the 5 mg/kg/day group, absolute and relative thymus weights in the 1.25 mg/kg/day group, and absolute thymus weight in the 5 mg/kg/day group were significantly decreased. Relative lung weights in 1.25, 2.5, and 5 mg/kg/day females and absolute lung weight in 1.25 mg/kg/day females were significantly increased. Biological significance of these changes is not clear.

In male rats, the total leukocyte count and the neutrophil count were significantly decreased at 5 mg/kg/day (Table 20). The reticulocyte count was decreased in 2.5 and 5.0 mg/kg/day males and females.

A mild significant elevation in BUN concentration was observed in the 5 mg/kg/day males (Table 20). Mild increases in BUN without increases in creatinine are most consistent with decreased water intake (i.e., dehydration). The globulin concentration was significantly decreased and albumin concentration significantly increased in the 5 mg/kg/day males, which resulted in an increase in the albumin:globulin ratio. In females, the albumin concentrations and albumin:globulin ratios were significantly elevated in the 2.5 and 5 mg/kg/day groups; total protein was also elevated in the 5 mg/kg/day group. In male rats, cholesterol concentrations were significantly decreased in all dose groups and triglycerides decreased in the 5 mg/kg/day group. In females, the cholesterol and triglyceride concentrations decreased at 5 mg/kg/day and at 2.5 and 5 mg/kg/day, respectively.

ALP and ALT activities were significantly elevated at 0.625 mg/kg/day and greater in male rats; AST activities were also elevated in several male dose groups (Table 20). In females, ALP and ALT activities were increased in the 2.5 and 5 mg/kg/day groups. Bile acid concentrations were significantly elevated in 5 mg/kg/day males and in 2.5 and 5 mg/kg/day females; direct bilirubin concentrations were elevated in the 5 mg/kg/day males and the 2.5 and 5 mg/kg/day females, and total bilirubin was elevated in the 5 mg/kg/day females.

Total T4 and free T4 concentrations were significantly decreased in all dose groups of male and female rats (Table 20). Total T3 concentrations were significantly decreased in the 0.625 mg/kg/day and higher male and female groups.

Male and female rats administered PFOS exhibited significant increases in expression of *Acox1*, *Cyp4a1*, *Cyp2b1,* and *Cyp2b2* compared to their respective controls, indicating significantly increased PPARα and CAR activity (Table 21). Males displayed a greater fold increase in *Cyp4a1* activity compared to controls than females compared to their controls, whereas CAR increases were more prominent in female rats compared to males. Expression of *Acox1* was the least sensitive of the genes evaluated in males, whereas *Cyp2b1* displayed the greatest fold changes compared to controls in both males and females.

Table 20. Selected Hematology and Clinical Chemistry Data for Rats in the 28-day Gavage Study of Perfluorooctane Sulfonic Acid[a]

	Vehicle Control	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day
Male						
n	10	10	10	10	10	10
Hematology						
Reticulocytes (10^3/μL)	181.1 ± 6.1	169.4 ± 11.6[b]	156.9 ± 8.0	154.2 ± 9.0[c]	140.0 ± 10.3**	104.4 ± 7.2**
Leukocytes (10^3/μL)	12.84 ± 0.61	14.18 ± 0.64	11.42 ± 0.64	11.15 ± 0.71	12.24 ± 0.63	10.72 ± 0.64*
Segmented Neutrophils (10^3/μL)	1.31 ± 0.11	1.28 ± 0.09[b]	1.17 ± 0.06	1.22 ± 0.13[c]	1.17 ± 0.05	0.97 ± 0.06*
Clinical Chemistry						
Urea Nitrogen (mg/dL)	17.7 ± 0.4	18.1 ± 1.0	16.8 ± 0.5	19.1 ± 0.8	18.0 ± 0.5	21.1 ± 0.7**
Total Protein (g/dL)	6.5 ± 0.1	6.4 ± 0.1	6.5 ± 0.1	6.7 ± 0.1	6.5 ± 0.1	6.6 ± 0.1
Albumin (g/dL)	4.3 ± 0.1	4.2 ± 0.0	4.2 ± 0.1	4.4 ± 0.0	4.4 ± 0.1	4.7 ± 0.0**
Globulin (g/dL)	2.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.1 ± 0.0	1.9 ± 0.1**
Albumin/Globulin Ratio	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	2.1 ± 0.0*	2.5 ± 0.1**
Direct Bilirubin (mg/dL)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.00**
Cholesterol (mg/dL)	115 ± 2	97 ± 3**	84 ± 2**	83 ± 4**	54 ± 3**	24 ± 3**
Triglycerides (mg/dL)	104 ± 6	156 ± 18	108 ± 10	118 ± 12	82 ± 10	24 ± 5**
Alanine Aminotransferase (IU/L)	48 ± 1	52 ± 2	66 ± 5**	56 ± 3**	75 ± 8**	71 ± 4**
Alkaline Phosphatase (IU/L)	187 ± 7	192 ± 5	217 ± 9*	218 ± 6**	215 ± 10*	252 ± 10**
Aspartate Aminotransferase (IU/L)	56 ± 2	60 ± 2	68 ± 4**	63 ± 2	68 ± 5*	61 ± 2
Bile Salt/Acids (μmol/L)	10.1 ± 3.8	12.3 ± 2.3	6.6 ± 1.2	10.8 ± 1.8	16.2 ± 1.7	34.7 ± 2.0**
Testosterone (ng/mL)	3.78 ± 0.75	5.81 ± 1.60	5.07 ± 1.72[b]	4.43 ± 1.72	5.58 ± 1.17	1.87 ± 1.37
Total Thyroxine (μg/dL)	3.51 ± 0.30	1.33 ± 0.19**	0.53 ± 0.09**	0.26 ± 0.07**	0.22 ± 0.04**	0.48 ± 0.07**
Free Thyroxine (ng/dL)	2.53 ± 0.22	0.95 ± 0.10**	0.47 ± 0.05**	0.40 ± 0.02**	0.36 ± 0.05**	0.33 ± 0.01**
Total Triiodothyronine (ng/dL)	87.37 ± 5.32	77.81 ± 5.44	60.63 ± 4.64**	57.50 ± 2.67**	55.35 ± 2.75**	50.00 ± 0.00**
Thyroid Stimulating Hormone (ng/mL)	20.39 ± 1.40	14.94 ± 1.74	14.79 ± 1.20[b]	23.33 ± 2.94	24.19 ± 3.38	18.90 ± 2.39

	Vehicle Control	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day
Female						
n	10	10	10	10	10	9
Hematology						
Reticulocytes ($10^3/\mu L$)	218.3 ± 25.2	208.5 ± 14.0	214.3 ± 15.9	164.7 ± 8.7	132.8 ± 11.2**	112.0 ± 10.3**
Clinical Chemistry						
Total Protein (g/dL)	6.4 ± 0.1	6.3 ± 0.1	6.6 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	6.9 ± 0.1**
Albumin (g/dL)	4.5 ± 0.1	4.4 ± 0.1	4.7 ± 0.1	4.7 ± 0.1*	4.8 ± 0.1**	5.0 ± 0.1**
Globulin (g/dL)	1.9 ± 0.0	1.8 ± 0.0	1.9 ± 0.1	2.0 ± 0.1	1.9 ± 0.1	1.9 ± 0.1
Albumin/Globulin Ratio	2.4 ± 0.1	2.4 ± 0.0	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.1*	2.6 ± 0.1*
Total Bilirubin (mg/dL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0*
Direct Bilirubin (mg/dL)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00*	0.05 ± 0.00**
Cholesterol (mg/dL)	105 ± 5	113 ± 3	100 ± 4	109 ± 6	93 ± 3	69 ± 5**
Triglycerides (mg/dL)	73 ± 9	75 ± 7	66 ± 3	64 ± 6	49 ± 3*	52 ± 7*
Alanine Aminotransferase (IU/L)	45 ± 2	44 ± 2	47 ± 1	48 ± 3	55 ± 2**	73 ± 3**
Alkaline Phosphatase (IU/L)	147 ± 5	142 ± 6	150 ± 6	170 ± 8	168 ± 8*	198 ± 10**
Bile Salt/Acids (μmol/L)	16.6 ± 2.8	12.5 ± 2.4	13.3 ± 1.9	21.0 ± 3.3	36.3 ± 4.3**	61.7 ± 7.4**
Total Thyroxine (μg/dL)	2.21 ± 0.24	1.11 ± 0.12**	0.55 ± 0.07**	0.33 ± 0.07**	0.35 ± 0.09**	0.38 ± 0.05**
Free Thyroxine (ng/dL)	1.74 ± 0.23	1.07 ± 0.09**	0.70 ± 0.03**	0.64 ± 0.05**	0.56 ± 0.05**	0.48 ± 0.03**
Total Triiodothyronine (ng/dL)	93.05 ± 5.04	81.40 ± 3.02	72.52 ± 4.27**	69.20 ± 3.63**	62.03 ± 1.78**	51.57 ± 1.43**
Thyroid Stimulating Hormone (ng/mL)	12.86 ± 0.73	14.76 ± 0.88	12.76 ± 0.85	13.25 ± 1.15	14.914 ± 1.95[b]	15.36 ± 0.73

*Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b]n = 9.
[c]n = 8.

Table 21. Hepatic Parameters for Rats in the 28-day Gavage Study of Perfluorooctane Sulfonic Acid[a]

	Vehicle Control	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day
Male						
n	10	10	10	10	10	10
Enzyme Activity						
Acyl-CoA Oxidase (nmol/min/mg)	1.987 ± 0.049	1.959 ± 0.076	1.736 ± 0.026	2.029 ± 0.049	2.793 ± 0.090**	6.053 ± 0.226**
Gene Expression						
Acox1	1.02 ± 0.06	1.02 ± 0.08	1.13 ± 0.06	1.82 ± 0.15**	2.92 ± 0.26**	5.36 ± 0.58**
Cyp4a1	1.04 ± 0.10	2.09 ± 0.18**	3.38 ± 0.25**	6.43 ± 0.51**	14.45 ± 1.56**	30.69 ± 3.62**
Cyp2b1	1.17 ± 0.21	5.87 ± 1.05**	11.61 ± 2.61**	88.63 ± 32.83**	135.34 ± 29.33**	408.63 ± 89.38**
Cyp2b2	1.22 ± 0.23	6.60 ± 1.01**	10.24 ± 0.91**	20.73 ± 2.022**	32.41 ± 3.23**	49.58 ± 4.69**
Female						
n	10	10	10	10	10	9
Gene Expression						
Acox1	1.02 ± 0.06	1.32 ± 0.05**	1.72 ± 0.15	1.41 ± 0.12**	2.05 ± 0.14**	3.10 ± 0.15**
Cyp4a1	1.03 ± 0.08	1.75 ± 0.12**	2.08 ± 0.20**	1.63 ± 0.16**	1.93 ± 0.18**	2.46 ± 0.24**
Cyp2b1	1.53 ± 0.44	32.47 ± 4.28**	99.63 ± 17.06**	260.11 ± 61.66**	671.64 ± 200.4**	1,226.9 ± 422.5**
Cyp2b2	1.56 ± 0.47	34.78 ± 6.76**	92.07 ± 13.79**	116.41 ± 11.01**	175.77 ± 17.26**	288.29 ± 21.56**

**Significantly different ($p \leq 0.01$) from the vehicle control group by Shirley's test.
[a]Data are presented as mean ± standard error. Gene expression data are presented as fold change values from the base-signal values.

Assessed male rats did not display any changes in sperm counts, motility, or testicular/epididymal weights (Table A-6). No treatment-related testicular or epididymal lesions were noted. Male serum testosterone levels assessed at necropsy in all treated groups were similar to the vehicle control level (Table 20).

The daily vaginal cytology data indicated that the female rats displayed similar amounts of time in each respective estrous stage (Table 22 and Table A-7; Figure A-3). Although summary statistics based on these data suggested that the proportion of time female rats spent in each stage of the cycle was similar across assessed groups, Markov analyses demonstrated that females in all assessed groups had a higher probability than the vehicle control group of transitioning to extended diestrus (Table 22 and Table A-7).

Table 22. Estrous Cycle Characterization for Female Rats in the 28-day Gavage Study of Perfluorooctane Sulfonic Acid[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day
n	10	10	10	10
Necropsy Body Wt. (g)	219 ± 3	222 ± 2	219 ± 4	215 ± 4
Number of Estrous Cycles	1.9 ± 0.18	2.3 ± 0.15	2.0 ± 0.00	1.9 ± 0.18
Estrous Cycle Length (days)	4.8 ± 0.20	4.9 ± 0.15	5.1 ± 0.15	5.2 ± 0.14
Estrous Stages (% of cycle)				
Diestrus	50.6	56.3	51.9	56.3
Proestrus	10.0	8.8	10.0	8.1
Estrus	34.4	30.0	35.0	33.8
Metestrus	3.8	5.0	3.1	1.9
Uncertain Diagnosis	1.3	0.0	0.0	0.0
Markov Analysis				
	Overall	**0.625 mg/kg/day**	**1.25 mg/kg/day**	**2.5 mg/kg/day**
Overall Test	p < 0.001	p < 0.001	p = 0.004	p = 0.001
Extended Diestrus	p < 0.001	p = 0.001	p = 0.028	p < 0.001

[a]Necropsy body weights, number of estrous cycles, and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle number and length). N indicates that the dose group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dose group had more departures from normal cycling than did the vehicle control group. Tests for equality of all transition matrices are shown in the vehicle control column (Markov analysis).

Pathology

The morphologic features of the lesions discussed in this section are presented in the Histopathologic Descriptions section following the Wyeth-14,643 results.

Liver: The incidences of hepatocyte hypertrophy were increased at 1.25, 2.5, and 5 mg/kg/day in males and females (Table 23). Hepatocyte hypertrophy was in general of minimal to mild severity. The incidences of hepatocyte, vacuolization cytoplasmic were increased in 2.5 and 5 mg/kg/day males, and in general, the lesion was of minimal severity. The incidences of

hepatocyte cytoplasmic alteration were increased in 1.25, 2.5, and 5 mg/kg/day females, and the lesion was in general of minimal severity.

Bone Marrow: The incidences of hypocellularity were significantly increased in the 1.25, 2.5, and 5 mg/kg/day males and 2.5 and 5 mg/kg/day females (Table 23). Hypocellularity was minimal to mild in severity.

Spleen: Extramedullary hematopoiesis is a normal occurrence in the spleen of rodents. There were significant increases in the incidences of decreased extramedullary hematopoiesis, in 1.25, 2.5, and 5 mg/kg/day males and females (Table 23). The severity of the lesion was minimal in all dose groups.

Table 23. Incidences of Selected Nonneoplastic Lesions in Rats in the 28-day Gavage Study of Perfluorooctane Sulfonic Acid

	Vehicle Control	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day
Male						
Liver[a]	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Hypertrophy[b]	0	0	0	3 (1.3)[c]	8** (1.3)	10** (2.1)
Hepatocyte, Vacuolization Cytoplasmic	0	0	0	0	2 (1.0)	4* (1.0)
Bone Marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hypocellularity	0	0	0	5* (1.0)	4* (1.0)	8** (1.0)
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Extramedullary Hematopoiesis, Decreased	1 (1.0)	1 (1.0)	2 (1.0)	7** (1.0)	8** (1.0)	10** (1.0)
Female						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration	0	0	0	3 (1.0)	5* (1.0)	10** (1.0)
Hepatocyte, Hypertrophy	0	0	0	2 (1.0)	3 (1.0)	10** (1.7)
Bone Marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hypocellularity	0	2 (1.0)	1 (1.0)	1 (1.0)	5* (1.0)	9** (1.8)
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Extramedullary Hematopoiesis, Decreased	2 (1.0)	3 (1.0)	3 (1.0)	8* (1.0)	10** (1.0)	10** (1.0)

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test.
**$p \leq 0.01$.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Wyeth-14,643

Wyeth-14,643 (WY) was used as a PPARα agonist (positive control) reference agent. All rats survived to scheduled euthanasia (Table 24). There were no significant treatment-related clinical observations in male or female rats.

The mean body weights of dose groups of males and females were within 10% of the respective vehicle control groups throughout the study (Table 24 and Figure 4).

In all treated male and female groups, compared to vehicle controls, there were dose-related and significant increases in the absolute and relative liver and right kidney weights (Table 25). Changes in the liver weights appear to correlate with histopathologic changes in the liver.

Absolute heart weight in 25 mg/kg/day males and absolute spleen weights in 6.25 and 25 mg/kg/day males were significantly decreased; biological significance of these changes is not clear.

Table 24. Mean Body Weights and Survival of Male Rats in the 28-day Gavage Study of Wyeth-14,643

	Vehicle Control		6.25 mg/kg/day			12.5 mg/kg/day			25 mg/kg/day		
Day	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
Male											
1	271	10	273	101	10	272	100	10	272	100	10
8	291	10	297	102	10	292	101	10	293	101	10
15	310	10	310	100	10	303	98	10	300	97	10
22	321	10	320	100	10	313	97	10	309	96	10
29	336	10	324	96	10	316	94	10	308	92	10
Female											
1	197	10	198	100	10	198	100	10	198	100	10
8	204	10	208	102	10	208	102	10	209	102	10
15	215	10	218	102	10	216	101	10	216	101	10
22	220	10	224	102	10	225	103	10	224	102	10
29	224	10	230	103	10	229	102	10	229	103	10

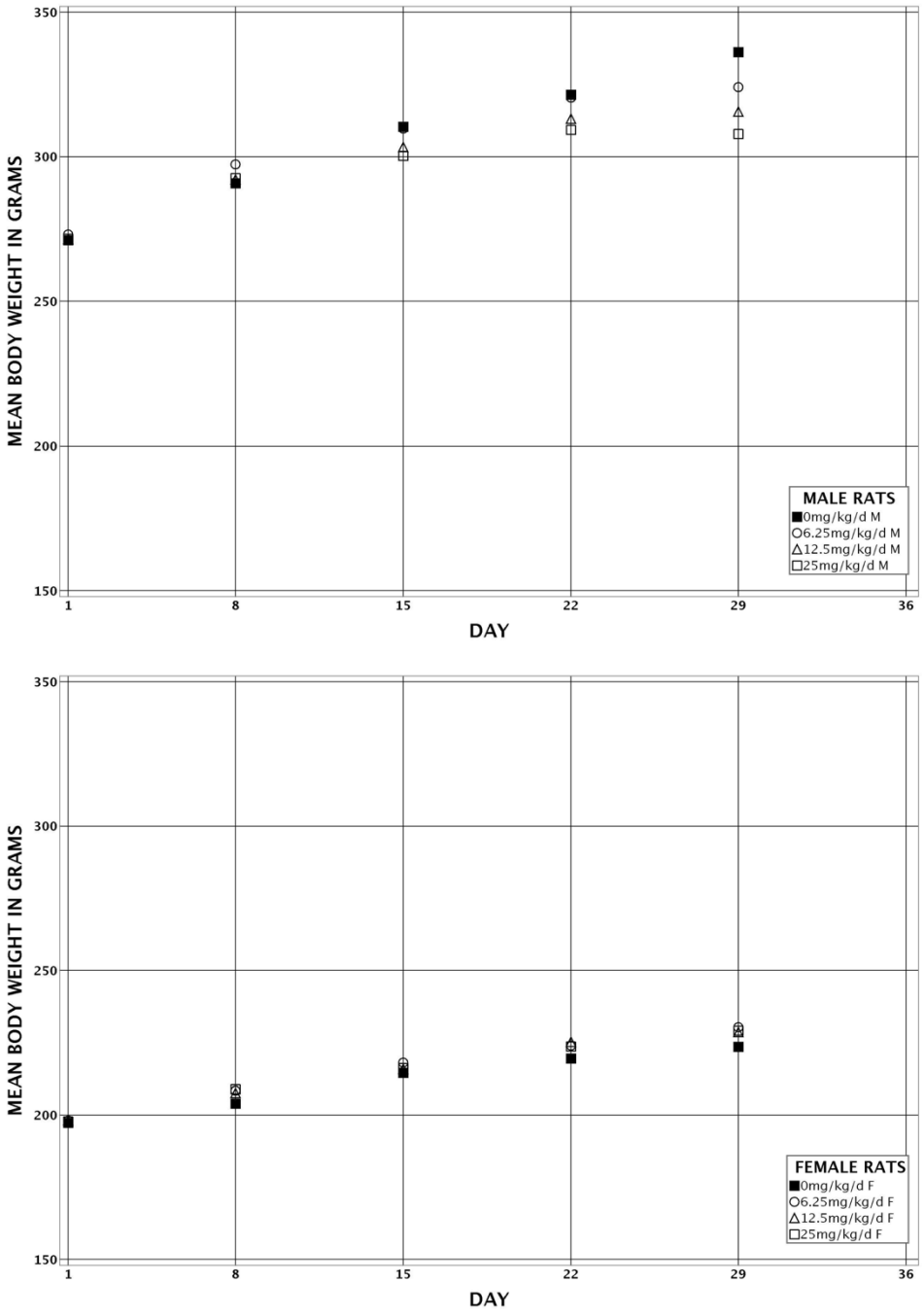


Figure 4. Growth Curves for Rats Administered Wyeth-14,643 by Gavage for 28 Days

Table 25. Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 28-day Gavage Study of Wyeth-14,643[a]

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
n	10	10	10	10
Male				
Necropsy Body Wt.	337 ± 4	324 ± 5	316 ± 5**	308 ± 5**
Heart				
Absolute	1.19 ± 0.02	1.17 ± 0.02	1.16 ± 0.02	1.10 ± 0.03*
Relative	3.54 ± 0.05	3.60 ± 0.06	3.67 ± 0.06	3.56 ± 0.04
R. Kidney				
Absolute	1.00 ± 0.02	1.10 ± 0.04*	1.13 ± 0.03**	1.13 ± 0.02**
Relative	2.98 ± 0.07	3.38 ± 0.08**	3.59 ± 0.08**	3.68 ± 0.05**
Liver				
Absolute	11.57 ± 0.21	15.35 ± 0.31**	15.97 ± 0.49**	16.56 ± 0.44**
Relative	34.33 ± 0.49	47.35 ± 0.46**	50.55 ± 1.22**	53.90 ± 1.67**
Spleen				
Absolute	0.723 ± 0.022	0.635 ± 0.017*	0.668 ± 0.022	0.631 ± 0.019**
Relative	2.14 ± 0.05	1.96 ± 0.06	2.11 ± 0.06	2.05 ± 0.05
Female				
Necropsy Body Wt.	224 ± 4	230 ± 4	229 ± 3	229 ± 5
R. Kidney				
Absolute	0.67 ± 0.01	0.74 ± 0.02**	0.75 ± 0.02**	0.76 ± 0.01**
Relative	3.02 ± 0.05	3.22 ± 0.08*	3.30 ± 0.09**	3.31 ± 0.04**
Liver				
Absolute	7.32 ± 0.19	10.25 ± 0.27**	11.07 ± 0.27**	11.98 ± 0.40**
Relative	32.78 ± 0.71	44.52 ± 0.90**	48.45 ± 1.05**	52.21 ± 1.14**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.
**$p \leq 0.01$.
[a]Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

There were several statistically significant changes, when compared to the respective vehicle control groups, in the hematology parameters of male and female rats. These changes, however, were mild and inconsistent and not considered toxicologically relevant.

BUN concentrations were significantly increased in all male dose groups (Table 26). Cholesterol concentrations were significantly decreased in all male dose groups. In all male dose groups, the total protein and globulin concentrations were significantly decreased, and the albumin concentrations were significantly increased; the combination of these changes resulted in significantly increased albumin:globulin ratios in male dose groups. In the female rats, globulin concentrations were significantly decreased in all dose groups, and total protein and albumin concentrations significantly increased in most dose groups; the combination of these changes resulted in significantly increased albumin:globulin ratios in all dose groups. ALT, ALP, and SDH activities were significantly increased in all male dose groups; AST was significantly increased in 25 mg/kg/day males. In addition, direct bilirubin concentrations were significantly increased in 25 mg/kg/day males and bile acid concentrations were significantly increased in all male dose groups. In female rats, ALP and SDH activities were significantly increased in all dose groups and ALT and AST activities were significantly increased in the 25 mg/kg/day group.

Free T4 and total T4 concentrations were significantly decreased in all male dose groups, and TSH was significantly decreased in the 25 mg/kg/day male group (Table 26). Additionally, testosterone concentration was significantly decreased in 25 mg/kg/day males. In females, T3 and TSH concentrations were significantly increased in most dose groups, and testosterone concentrations were significantly increased at 6.25 and 25 mg/kg/day.

There were significant increases in expression of *Acox1*, *Cyp4a1*, *Cyp2b1*, and *Cyp2b2* compared to controls, indicating significantly increased PPARα and CAR activities in treated male and female rats (Table 27). Males displayed a greater fold increase in *Cyp4a1* activity compared to controls than females compared to their controls, whereas CAR increases compared to controls were more prominent in female rats than males.

Male rats administered 6.25, 12.5, or 25 mg/kg/day for 28 days displayed lower testicular spermatid counts (9%, 14%, 13%, respectively) relative to vehicle controls (Table 28). When normalized to total testicular weight, counts were slightly lower in the dose groups, with the 12.5 and 25 mg/kg/day groups exhibiting lower (11% and 10%) counts relative to vehicle control. These differences did not attain statistical significance, but the trend was significant. Wyeth-14,643 administration did not affect motility or testis weight. Cauda epididymal sperm counts (both total and normalized to total weight) were generally lower (22% to 25%) in the dose rats; however, none of these changes attained statistical significance. Epididymis weights in treated males were 10% to 12% lower than vehicle controls with the observed decrease in 25 mg/kg/day males attaining statistical significance. The trend test was also significant. One rat in the 25 mg/kg/day group displayed histopathologic findings in the testis (germinal epithelium degeneration) and epididymis duct (exfoliated germ cell). Testosterone measured at time of necropsy was significantly lower (61%) in the 25 mg/kg/day group (positive trend test).

Table 26. Selected Clinical Chemistry Data for Rats in the 28-day Gavage Study of Wyeth-14,643[a]

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
n	10	10	10	10
Male				
Urea Nitrogen (mg/dL)	18.6 ± 0.7	20.2 ± 0.5*	21.3 ± 0.5**	23.1 ± 0.6**
Total Protein (g/dL)	6.59 ± 0.05	6.24 ± 0.13*	6.37 ± 0.05*	6.25 ± 0.07**
Albumin (g/dL)	4.30 ± 0.03	4.74 ± 0.06**	4.78 ± 0.04**	4.86 ± 0.09**
Globulin (g/dL)	2.29 ± 0.03	1.50 ± 0.07**	1.59 ± 0.04**	1.39 ± 0.04**
Albumin/Globulin Ratio	1.88 ± 0.02	3.22 ± 0.13**	3.02 ± 0.08**	3.53 ± 0.14**
Cholesterol (mg/dL)	125.0 ± 2.7	106.6 ± 5.0**	116.2 ± 4.0*	101.8 ± 3.5**
Alanine Aminotransferase (IU/L)	52.20 ± 1.32	62.80 ± 4.29*	59.50 ± 2.36*	62.70 ± 2.10**
Alkaline Phosphatase (IU/L)	202.8 ± 7.5	286.2 ± 12.2**	325.1 ± 14.7**	386.5 ± 31.2**
Aspartate Aminotransferase (IU/L)	60.00 ± 1.26	64.70 ± 3.45	62.50 ± 1.90	70.00 ± 3.35*
Sorbitol Dehydrogenase (IU/L)	5.7 ± 0.4	9.9 ± 1.4**	7.9 ± 0.5**	8.4 ± 0.4**
Direct Bilirubin (mg/dL)	0.024 ± 0.003	0.021 ± 0.002	0.025 ± 0.002	0.030 ± 0.002*
Bile Acids (µmol/L)	11.8 ± 1.6	40.9 ± 6.5**	73.1 ± 11.2**	83.2 ± 9.5**
Total Triiodothyronine (ng/dL)	106.35 ± 5.53	113.62 ± 4.21	102.40 ± 3.39	103.95 ± 4.98
Total Thyroxine (µg/dL)	3.58 ± 0.13	2.90 ± 0.15**	2.94 ± 0.20*	2.78 ± 0.17**
Free Thyroxine (ng/dL)	2.35 ± 0.09	1.43 ± 0.13**	1.41 ± 0.14**	1.31 ± 0.13**
Thyroid Stimulating Hormone (ng/mL)	18.56 ± 1.16	21.46 ± 3.59	15.64 ± 2.13	13.07 ± 1.01*
Testosterone	4.03 ± 0.48	3.14 ± 0.91	4.37 ± 1.29	1.56 ± 0.39*
Female				
Total Protein (g/dL)	6.37 ± 0.08	6.54 ± 0.05	6.85 ± 0.12**	6.85 ± 0.08**
Albumin (g/dL)	4.46 ± 0.05	5.03 ± 0.05**	5.20 ± 0.08**	5.20 ± 0.05**
Globulin (g/dL)	1.91 ± 0.05	1.51 ± 0.04**	1.65 ± 0.05*	1.65 ± 0.05*
Albumin/Globulin Ratio	2.34 ± 0.05	3.35 ± 0.10**	3.17 ± 0.08**	3.17 ± 0.09**
Alanine Aminotransferase (IU/L)	41.80 ± 1.24	49.50 ± 3.85	46.80 ± 1.85	54.80 ± 2.58**
Alkaline Phosphatase (IU/L)	142.6 ± 6.9	181.7 ± 10.2**	179.2 ± 5.4**	217.3 ± 11.4**
Aspartate Aminotransferase (IU/L)	55.30 ± 1.08	64.70 ± 8.15	56.50 ± 1.70	61.90 ± 1.55*
Sorbitol Dehydrogenase (IU/L)	4.2 ± 0.5	9.9 ± 2.4**	7.7 ± 0.7**	8.3 ± 0.6**
Total Triiodothyronine (ng/dL)	65.66 ± 2.47	71.86 ± 4.31	83.09 ± 4.79**	83.57 ± 3.72**
Total Thyroxine (µg/dL)	1.79 ± 0.13	1.99 ± 0.16	2.28 ± 0.15	1.70 ± 0.12
Free Thyroxine (ng/dL)	1.13 ± 0.08	1.44 ± 0.10	1.67 ± 0.14	1.34 ± 0.09
Thyroid Stimulating Hormone (ng/mL)	10.07 ± 0.51[b]	16.19 ± 2.03**	14.96 ± 1.71**	17.21 ± 1.25**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b]n = 9.

Table 27. Hepatic Parameters for Rats in the 28-day Gavage Study of Wyeth-14,643[a]

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
n	10	10	10	10
Male				
Enzyme Activity				
Acyl-CoA Oxidase (nmol/min/mg)	2.028 ± 0.044	11.731 ± 0.692**	14.560 ± 1.027**	17.370 ± 0.592**
Gene Expression				
Acox1	1.05 ± 0.10	3.54 ± 0.60**	4.77 ± 0.55**	6.86 ± 0.44**
Cyp4a1	1.06 ± 0.10	29.92 ± 4.63**	33.96 ± 2.45**	45.82 ± 3.31**
Cyp2b1	1.21 ± 0.21	3.77 ± 0.94**	3.69 ± 0.67**	4.45 ± 0.95**
Cyp2b2	1.27 ± 0.28	2.32 ± 0.23**	1.93 ± 0.20*	2.43 ± 0.19**
Female				
Gene Expression				
Acox1	1.01 ± 0.05	3.11 ± 0.26**	4.08 ± 0.27**	5.65 ± 0.49**
Cyp4a1	1.02 ± 0.07	2.90 ± 0.52**	3.85 ± 1.12**	7.26 ± 1.22**
Cyp2b1	1.18 ± 0.24	24.05 ± 4.59**	28.82 ± 3.36**	38.79 ± 4.74**
Cyp2b2	1.18 ± 0.21	19.59 ± 2.67**	29.41 ± 1.48**	30.41 ± 3.22**

*Significantly different ($p \leq 0.05$) from the vehicle control group by Shirley's test.
**$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Gene expression data are presented as fold change values from the base-signal values.

Table 28. Summary of Reproductive Tissue Evaluations for Male Rats in the 28-day Gavage Study of Wyeth-14,643[a]

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
n	10	10	10	10
Weights (g)				
Necropsy Body Wt.	337 ± 4	324 ± 5	316 ± 5	308 ± 5**
L. Cauda Epididymis	0.230 ± 0.035**	0.197 ± 0.007	0.188 ± 0.004	0.179 ± 0.006
L. Epididymis	0.584 ± 0.037*	0.519 ± 0.008	0.524 ± 0.012	0.512 ± 0.011*
L. Testis	1.840 ± 0.029	1.786 ± 0.040	1.780 ± 0.043	1.774 ± 0.036
Spermatid Measurements				
Spermatid Heads (10^6/testis)	294.3 ± 13.6	269.2 ± 13.7	253.9 ± 13.7	255.8 ± 11.5
Spermatid Heads (10^6/g testis)	159.8 ± 6.3*	150.7 ± 6.9	141.9 ± 4.8	144.4 ± 6.4
Epididymal Spermatozoal Measurements				
Sperm Motility (%)	88.5 ± 0.5	87.9 ± 0.3	87.9 ± 0.3	88.2 ± 0.5
Sperm (10^6/g cauda epididymis)	148.4 ± 17.4	111.0 ± 8.2	115.3 ± 7.8	116.3 ± 8.4
Sperm (10^6/cauda epididymis)	671 ± 46	562 ± 31	616 ± 44	643 ± 33
Testosterone (ng/mL)	4.03 ± 0.48	3.14 ± 0.91	4.37 ± 1.29	1.56 ± 0.39*

**Significantly different ($p \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test.
*$p \leq 0.01$.
[a]Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Williams' or Dunnett's test (cauda epididymis and testis weights) or Shirley's or Dunn's test (spermatid and epididymal spermatozoal measurements).

Summary statistics based on the daily vaginal cytology data suggested that the proportion of time female rats spent in each stage of the cycle was similar across assessed groups (Table 29 and Table A-8; Figure A-4). However, Markov analyses demonstrated that females in all assessed groups administered Wyeth-14,643 had a higher probability than the vehicle control group of transitioning to extended diestrus (Table 29 and Table A-8). Under the conditions of this study, these data indicate that Wyeth-14,643 via oral gavage exhibits the potential to be a reproductive toxicant in female Sprague Dawley rats based on altered estrus cyclicity (extended diestrus).

Table 29. Estrous Cycle Characterization for Female Rats in the 28-day Gavage Study of Wyeth-14,643[a]

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
n	10	10	9	10
Necropsy Body Wt. (g)	224 ± 4	230 ± 4	229 ± 3[b]	229 ± 5
Number of Estrous Cycles	2.0 ± 0.15	2.0 ± 0.0	1.9 ± 0.11	1.8 ± 0.13
Estrous Cycle Length (days)	5.0 ± 0.16	5.0 ± 0.09	4.8 ± 0.26	5.1 ± 0.16
Estrous Stages (% of cycle)				
Diestrus	55.6	61.9	58.1	61.9
Proestrus	4.4	3.8	12.5	6.3
Estrus	38.1	32.5	25.6	28.8
Metestrus	1.9	1.9	3.1	3.1
Uncertain Diagnosis	0.0	0.0	0.6	0.0
Markov Analysis				
	Overall	**6.25 mg/kg/day**	**12.5 mg/kg/day**	**25 mg/kg/day**
Overall Tests	p = 0.002	p = 0.085	p < 0.001N	p = 0.983
Extended Diestrus	p < 0.001	p = 0.005	p = 0.001	p = 0.009

[a]Necropsy body weights, number of estrous cycles, and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle number and length). N indicates that the dose group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dose group had more departures from normal cycling than did the vehicle control group. Tests for equality of all transition matrices are shown in the vehicle control column (Markov analysis).
[b]n = 10.

Pathology

The morphologic features of the lesions discussed in this section are presented in the Histopathologic Descriptions section following these results.

Liver: The incidences of hepatocyte hypertrophy were significantly increased in all dose groups of males and in 25 mg/kg/day females (Table 30). Hepatocyte hypertrophy was in general of minimal to mild severity in males and minimal in females. The average severity increased with increasing dose in males. The incidences of hepatocyte cytoplasmic alteration were significantly increased in all dose groups of males and females, and in general, the lesion was of minimal to marked severity in males and minimal to mild in females. The average severity increased with increasing dose.

Table 30. Incidences of Nonneoplastic Lesions of the Liver in Rats in the 28-day Gavage Study of Wyeth-14,643

	Vehicle Control	6.25 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day
Male				
Number Examined Microscopically	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration[a]	0	9** (1.7)[b]	10** (2.2)	10** (4.0)
Hepatocyte, Hypertrophy	0	5* (1.4)	10** (1.5)	10** (2.0)
Female				
Number Examined Microscopically	(10)	(10)	(10)	(10)
Hepatocyte, Cytoplasmic Alteration	0	7** (1.0)	10** (1.2)	10** (1.9)
Hepatocyte, Hypertrophy	0	0	2 (1.0)	8** (1.1)

*Significantly different ($p \leq 0.05$) from the vehicle control group by the Fisher exact test.
**$p \leq 0.01$.
[a]Number of animals with lesion.
[b]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Histopathologic Descriptions

In general, the histopathologic changes, when present, in the liver, bone marrow, nose, forestomach, thymus, kidney, and spleen were morphologically similar across the studies. However, the severities of the lesions varied among the studies.

Liver: Hepatocyte hypertrophy (Figure 5, Figure 6, Figure 7, and Figure 8) was characterized by an increase in the size of primarily centrilobular hepatocytes with cytoplasmic granule accumulation or an increase in homogenous eosinophilic cytoplasm (in the absence of distinct eosinophilic cytoplasmic granules). Hepatocyte hypertrophy was graded as minimal (Grade 1) when hepatocytes were enlarged in occasional lobules (up to 10% of the liver involved), mild (Grade 2) when 11% to 33% of the liver was involved, moderate (Grade 3) when 34% to 67% of the liver was involved, and marked (Grade 4) when greater than 67% of the liver was involved.

Hepatocyte cytoplasmic alteration (Figure 9 and Figure 10) was characterized by an accumulation of eosinophilic granules within the cytoplasm of centrilobular hepatocytes. A grade of minimal (Grade 1) was used when eosinophilic granules were present within occasional centrilobar hepatocytes (up to 10% of the liver involved); mild (Grade 2) when 11% to 33% of the liver was involved, with most or all centrilobular zones affected; moderate (Grade 3) when 34% to 67% of the liver was involved, with widespread centrilobular and midzonal hepatocytes affected, and marked (Grade 4) when greater than 67% of the liver was involved.

Cytoplasmic vacuolation was characterized by the presence of small round cytoplasmic vacuoles within the hepatocytes. A grade of minimal (Grade 1) was used when cytoplasmic vacuoles were present within occasional centrilobular to midzonal hepatocytes (up to 10% of the liver involved), mild (Grade 2) when 11% to 33% of the liver was involved, moderate (Grade 3) when 34% to 67 % of the liver was involved, and marked (Grade 4) when greater than 67% of the liver was involved.

Necrosis was characterized by one small area of coagulative necrosis containing hepatocytes with bright eosinophilic cytoplasm, indistinct cell borders, loss of nuclei, and infiltration of small numbers of macrophages and neutrophils. A severity grade of minimal was used for this lesion.

Bone Marrow: Bone marrow hypocellularity was characterized by a decrease in both erythroid and myeloid cell lines and adipocytes (fat cells) in the bone marrow appeared to be more numerous due to loss of hematopoietic cells. Minimal bone marrow hypocellularity was diagnosed when the bone marrow contained approximately 10% to 15% more adipocytes and a corresponding reduction in the number of hematopoietic cells than concurrent vehicle controls. Mild hypocellularity was diagnosed when the bone marrow contained 16% to 30% more adipocytes and a corresponding reduction in the number of hematopoietic cells than concurrent vehicle controls.

Nose: Olfactory epithelium degeneration (Figure 11 and Figure 12) was characterized by segmentally decreased nuclear density and decreased height of the olfactory epithelium due to loss of the olfactory epithelial cells. In the affected segments, the remaining epithelial cells had lost the surface cilia/microvillus border and contained cytoplasmic vacuoles, and there were scattered pyknotic nuclei within the epithelium. Olfactory epithelium degeneration was diagnosed as minimal (Grade 1) when epithelium on up to 25% of the nasal turbinates was affected, as mild (Grade 2) when epithelium on 26% to 50% of the nasal turbinates was affected, as moderate (Grade 3) when epithelium on 51% to 75% of the nasal turbinates was affected, and as marked (Grade 4) when epithelium on greater than 75% of the nasal turbinates was affected.

Olfactory epithelium hyperplasia (Figure 13, Figure 14, Figure 15, and Figure 16) was characterized by multifocal to linear areas of proliferation and piling up of the olfactory epithelial cells resulting in disorganization and increased thickness of the epithelium. In some affected segments, focal proliferations of basal epithelial cells extended into the underlying propria effacing the normally distinct border between olfactory epithelium and the underlying lamina propria. A grade of minimal (Grade 1) was used when up to 15% of the olfactory epithelium in Level III was involved, and mild (Grade 2) was used when 16% to 50% of the Level III olfactory epithelium was hyperplastic. No grade higher than minimal was recorded for olfactory epithelial hyperplasia.

Olfactory epithelium suppurative inflammation (Figure 17) was characterized by aggregates of neutrophils and cellular debris within the nasal passages and within the epithelium and lamina propria of the ethmoid turbinates. A grade of minimal (Grade 1) was used when up to 25% of the nasal cavity lumen contained neutrophils, mild (Grade 2) when 26% to 50% of the nasal cavity lumen contained neutrophils, moderate (Grade 3) when 51% to 75% of the nasal cavity lumen contained neutrophils, and marked (Grade 4) when greater than 75% of the nasal cavity contained neutrophils.

Olfactory epithelium necrosis (Figure 18) was often limited to the superficial olfactory epithelial cells. Affected cells were swollen, contained condensed, darkly stained nuclei and had hypereosinophilic cytoplasm. Exfoliated necrotic cells were often present in the lumen admixed with degenerate neutrophils and cellular debris. Olfactory epithelium necrosis was diagnosed as minimal (Grade 1) when epithelium on up to 25% of the nasal turbinates was affected, as mild (Grade 2) when epithelium on 26% to 50% of the nasal turbinates was affected, as moderate

(Grade 3) when epithelium on 51% to 75% of the nasal turbinates was affected, and as marked (Grade 4) when epithelium on greater than 75% of the nasal turbinates was affected.

Stomach, Forestomach: Epithelium hyperplasia was characterized by a focal area of increased cell numbers in the mucosa resulting in an increased thickness and folding of the mucosa. The epithelial cells maintained normal orientation, and atypia was not present. The change was diagnosed as minimal (Grade 1) when a focal area was involved with two times the thickness of normal mucosa, and as mild (Grade 2) when the thickness was three to four times the normal thickness.

Thymus: Atrophy was characterized by thinning of the cortex and loss of the corticomedullary demarcation. The severity of this finding was diagnosed as minimal (Grade 1) when there was up to a 25% reduction in cortex, mild (Grade 2) when there was a 26% to 50% reduction of the cortex, moderate (Grade 3) when there was a 51% to 75% reduction of the cortex, and marked (Grade 4) when there was more than 75% reduction in the cortex and loss of the corticomedullary junction.

Kidney: Papilla necrosis (Figure 19, Figure 20, Figure 21, and Figure 22) was characterized by a region of well-defined loss of differential staining with sloughing of renal tubule/collecting duct epithelium from the basement membrane and accumulation of fibrin within the papillary interstitium. Variably, scattered degenerate neutrophils were present within and just adjacent to these foci. Papillary necrosis was diagnosed as minimal (Grade 1) when greater than 10 contiguous tubules were affected and as mild (Grade 2) when 11 to 25 contiguous tubules were affected.

Spleen: Extramedullary hematopoiesis, decreased was characterized by absence of or presence of very rare erythroid elements as compared to the presence of normal, minimal to moderate extramedullary hematopoiesis in vehicle controls.

Genetic Toxicology

PFBS and PFOS were tested in assays that measure the potential for induction of mutations in bacteria (*Salmonella typhimurium* strains TA100 and TA98 and *Escherichia coli* strain WP2 *uvrA*/pKM101). Chromosomal damage in erythrocyte precursor cells in the peripheral blood was determined in male and female rats from all four of the 28-day studies. Although the damage is induced in the erythroblast population in the bone marrow, it is measured as micronuclei in immature red blood cells (reticulocytes, RET; polychromatic erythrocytes, PCEs) in peripheral blood.

PFBS (concentration range of 50 to 5,000 μg/plate) was judged to be equivocal in *S. typhimurium* strain TA98 due to a mix of negative and positive results that were observed both in the presence and absence of S9, and an equivocal result in the presence of S9 (Table B-1). No mutagenic activity was seen in *S. typhimurium* strain TA100 using a similar concentration range or in *E. coli* strain WP2 *uvrA*/pKM101 (concentration range of 50 to 1,000 μg/plate). No mutagenic activity was seen for PFOS in *S. typhimurium* TA100 or TA98 (concentration range of 50 to 5,000 μg/plate) or *E. coli* strain WP2 *uvrA*/pKM101 (concentration range of 100 to 10,000 μg/plate) (Table B-2).

In rats, the reticulocyte population is the only red blood cell population that can be accurately assessed for micronucleus frequency in peripheral blood due to efficient splenic scavenging of damaged erythrocytes soon after they emerge from the bone marrow. In females administered PFOS, the frequency of micronucleated PCEs was significantly increased at the highest dose of 5 mg/kg/day (Table B-5). The effect at this dose also resulted in a significant trend test, therefore meeting statistical criteria for a positive result (both a significant trend and at least one significant dose group). However, the frequency at the highest dose was within the historical control range, and therefore the result was judged to be equivocal. No increases in the frequencies of micronucleated erythrocytes (either immature or mature) were observed in peripheral blood samples from males administered PFOS. However, significant, generally dose-dependent decreases in the percentage of PCEs in the peripheral blood of male and female rats were observed, suggesting that the bone marrow was a target for PFOS-induced cytotoxicity.

No increases in the frequencies of micronucleated erythrocytes (either immature or mature) were observed in peripheral blood samples from male or female rats administered PFBS (concentration range of 62.6 to 500 mg/kg/day), PFHxSK (concentration ranges of 0.625 to 10 [males] or 3.12 to 50 [females] mg/kg/day), or Wyeth-14,643 (concentration range of 6.25 to 25 mg/kg/day) for 28 days (Table B-3, Table B-4, and Table B-6, respectively). However, PFBS caused significant, dose-dependent decreases in the percentage of PCEs in the peripheral blood of both sexes, as did PFHxSK in males, suggesting that the bone marrow was a target for cytotoxicity induced by these chemicals.

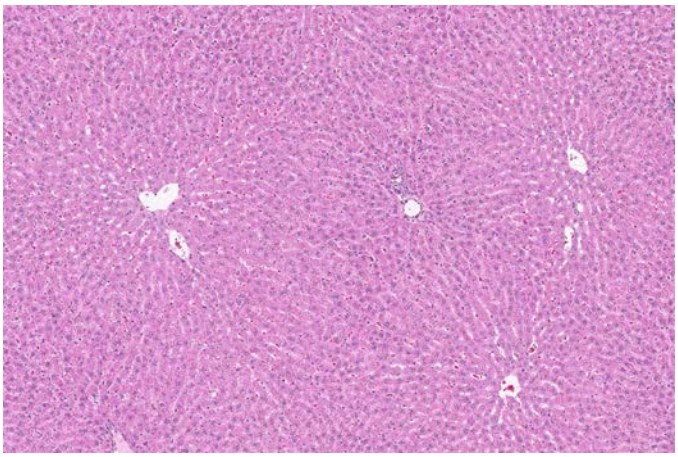


Figure 5. Normal Liver of a Male Sprague Dawley Vehicle Control Rat from the 28-day Gavage Study of Perfluorobutane Sulfonic Acid (H&E)

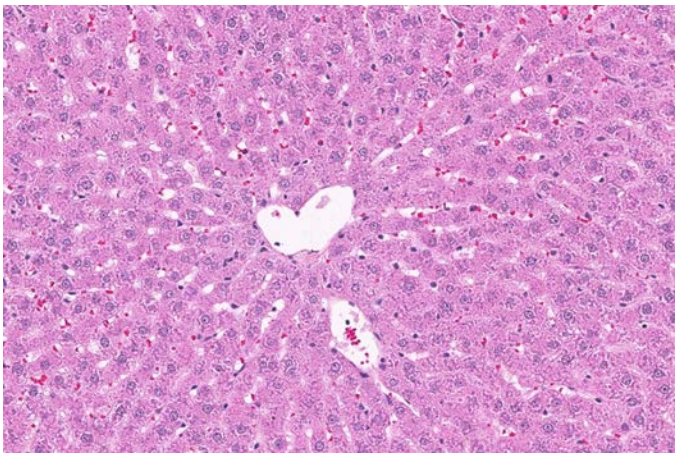


Figure 6. Higher Magnification of Figure 5 Demonstrating Normal Hepatocytes (H&E)

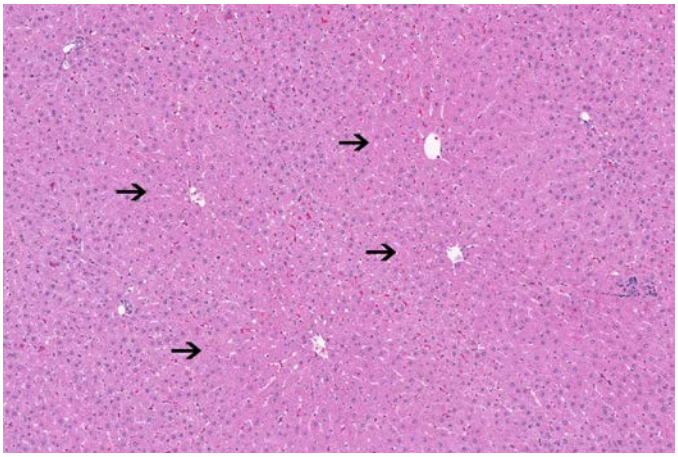


Figure 7. Centrilobular Areas (Arrows) of Hepatocyte Hypertrophy in a Male Sprague Dawley Rat Administered 500 mg/kg/day Perfluorobutane Sulfonic Acid for 28 Days (H&E)

Hepatocytes are more eosinophilic in these areas.

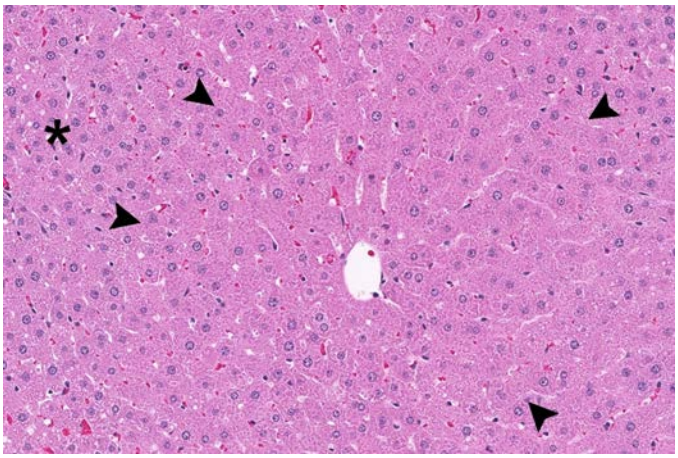


Figure 8. Higher Magnification of Figure 7 Demonstrating Enlarged Hepatocytes with Eosinophilic Cytoplasm (H&E)

In the hypertrophied area (marked by arrowheads), hepatocyte nuclei are farther apart compared to normal area (asterisk).

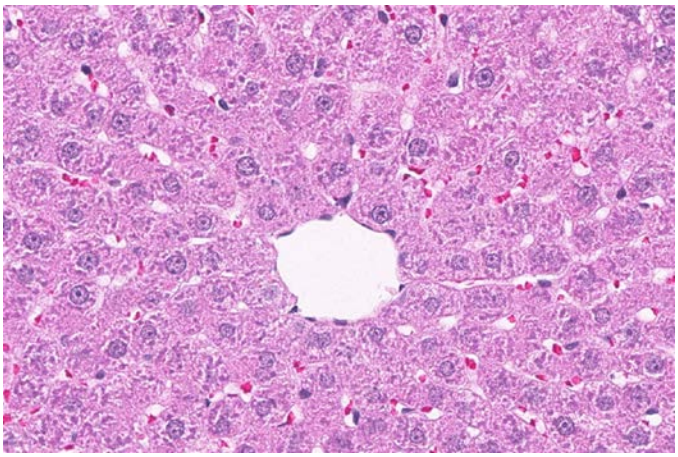


Figure 9. Normal Hepatocytes in the Liver of a Male Sprague Dawley Vehicle Control Rat from the 28-day Gavage Study of Perfluorobutane Sulfonic Acid (H&E)

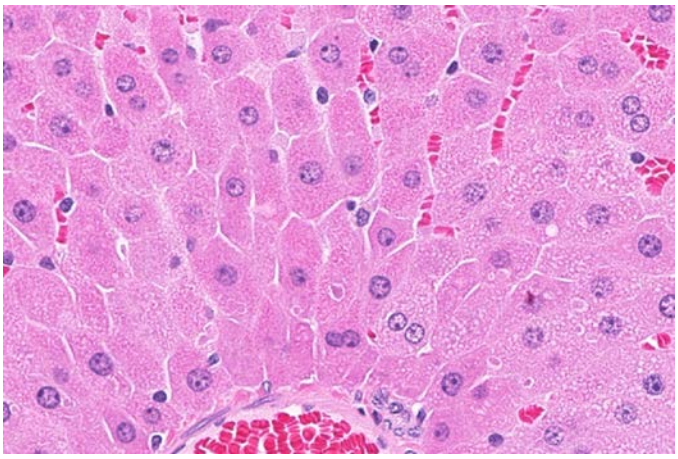


Figure 10. Cytoplasmic Alteration and Hypertrophy Characterized by Enlarged Hepatocytes with Eosinophilic Granular Cytoplasm in a Male Sprague Dawley Rat Administered 1,000 mg/kg/day Perfluorobutane Sulfonic Acid for 28 Days (H&E)

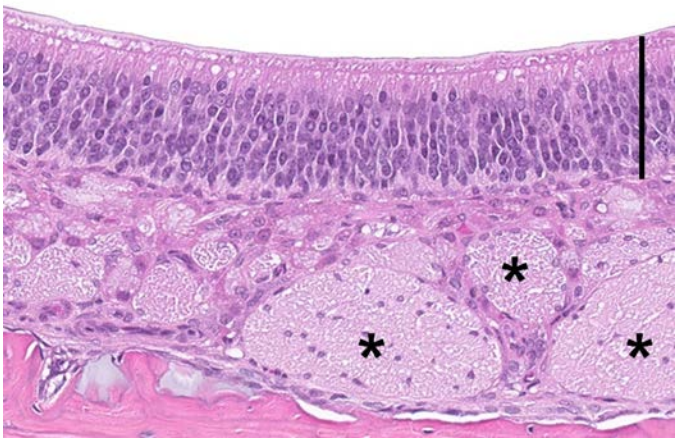


Figure 11. Normal Olfactory Epithelium in the Nose of a Male Sprague Dawley Vehicle Control Rat from the 28-day Gavage Study of Perfluorobutane Sulfonic Acid (H&E)

Asterisks indicate normal olfactory nerves.

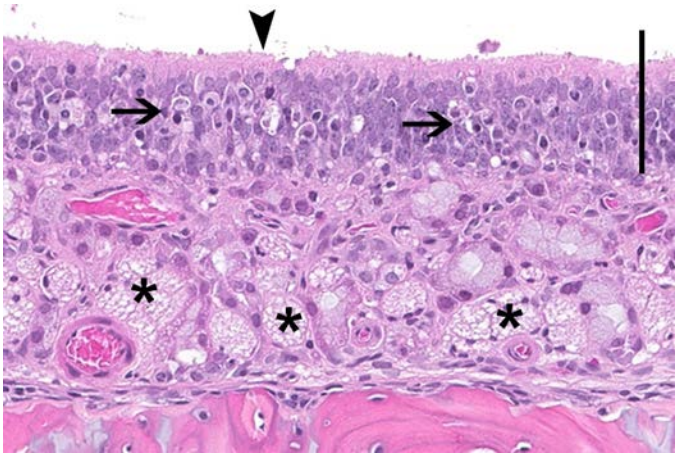


Figure 12. Olfactory Epithelium Degeneration in a Male Sprague Dawley Rat Administered 1,000 mg/kg/day Perfluorobutane Sulfonic Acid for 28 Days (H&E)

Characterized by decreased nuclear density, decreased height of the olfactory epithelium due to the loss of cells (compare the black bar with the black bar in Figure 11) and atrophy of olfactory nerves (asterisks; compare this to asterisks in Figure 11). The surface cilia/microvillus border (arrowhead) is lost and there are pyknotic nuclei (arrows) within the epithelium.

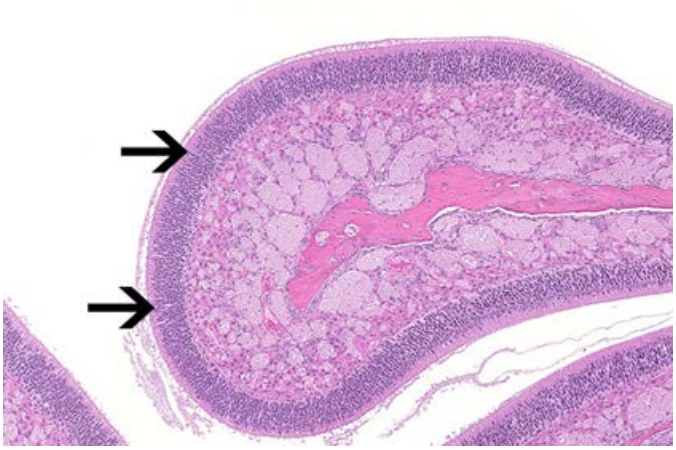


Figure 13. Normal Olfactory Epithelium (Arrows) in a Control Female Sprague Dawley Rat (H&E)

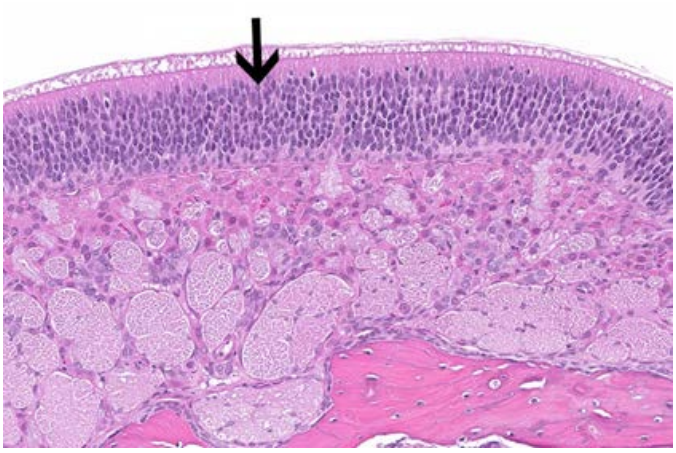


Figure 14. Magnified Area from the Section in Figure 15 (H&E) Showing Normal Olfactory Epithelium (Arrow)

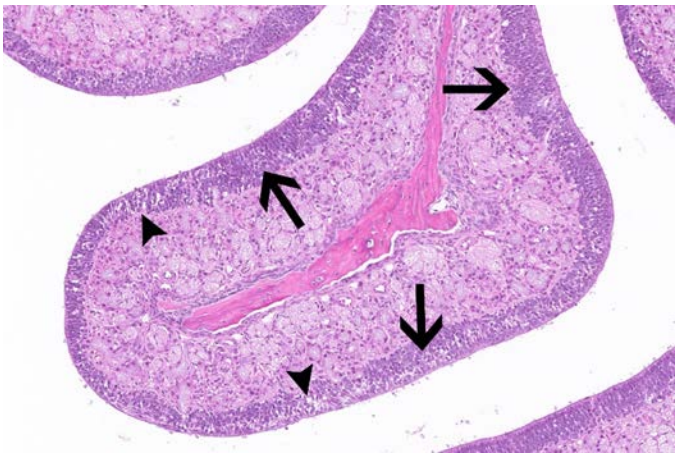


Figure 15. Olfactory Epithelium Degeneration (Arrowheads), Associated with Areas of Olfactory Epithelium Hyperplasia (Arrows) in a Female Sprague Dawley Rat Administered 500 mg/kg/day Perfluorobutane Sulfonic Acid for 28 Days (H&E)

Hyperplastic areas are characterized by increased thickness of the epithelium. Compare this to normal olfactory epithelium in Figure 13.

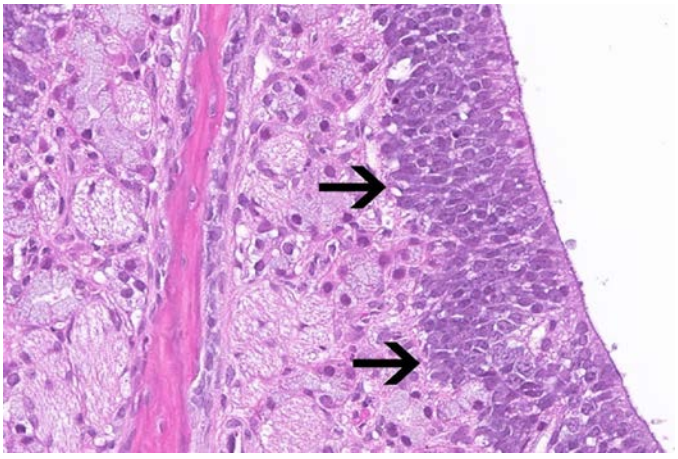


Figure 16. Magnified Area from the Section in Figure 15 (H&E)

The thickness of epithelium is increased, epithelium is disorganized and basal cells extend into the underlying propria (arrows). Compare this to the normal olfactory epithelium in Figure 14.

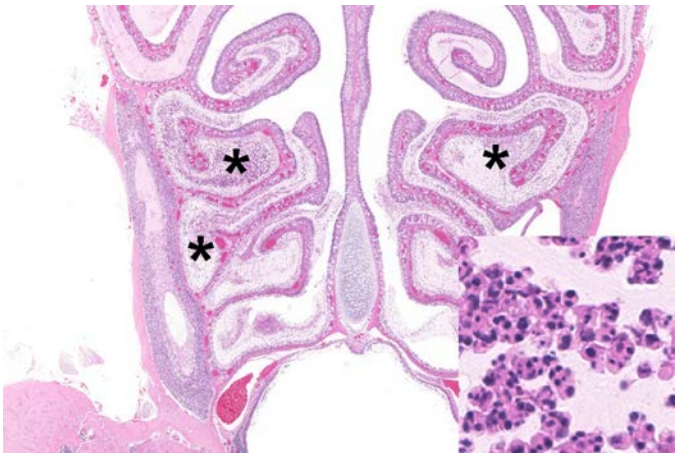


Figure 17. Suppurative Inflammation in the Olfactory Epithelium Region (Asterisks) in a Female Sprague Dawley Rat Administered 1,000 mg/kg/day Perfluorobutane Sulfonic Acid for 28 Days (H&E)

Inflammation is characterized by the presence of degenerate neutrophils and cellular debris (asterisks and inset) within the nasal passages.

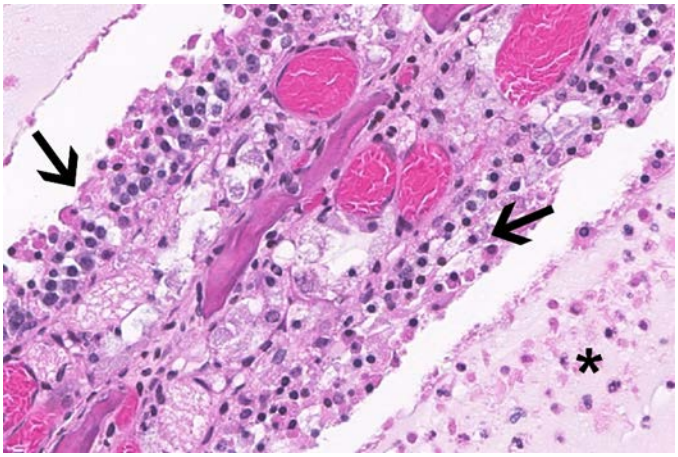


Figure 18. Necrosis of Olfactory Epithelium in a Male Sprague Dawley Rat Administered 1,000 mg/kg/day Perfluorobutane Sulfonic Acid for 28 Days (H&E)

Necrosis is characterized by the presence of cells with hypereosinophilic cytoplasm, condensed nuclei, cellular debris with disorganized epithelium (arrows), and the presence of exfoliated cells and degenerate neutrophils in the nasal passage (asterisk).

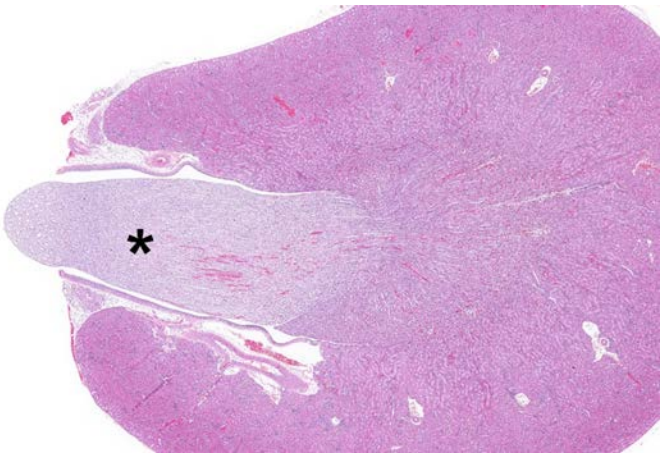


Figure 19. Normal Renal Papilla (Asterisk) in a Vehicle Control Female Sprague Dawley Rat from the 28-day Gavage Study of Perfluorobutane Sulfonic Acid (H&E)

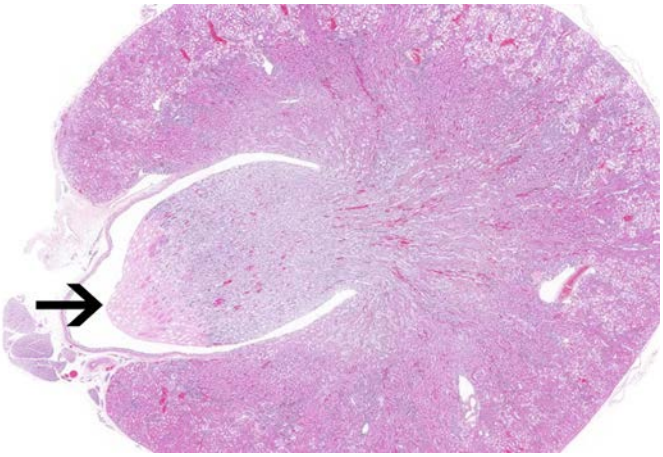


Figure 20. Necrosis of Renal Papilla (Arrow) in a Female Sprague Dawley Rat Administered 1,000 mg/kg/day Perfluorobutane Sulfonic Acid for 28 days (H&E)

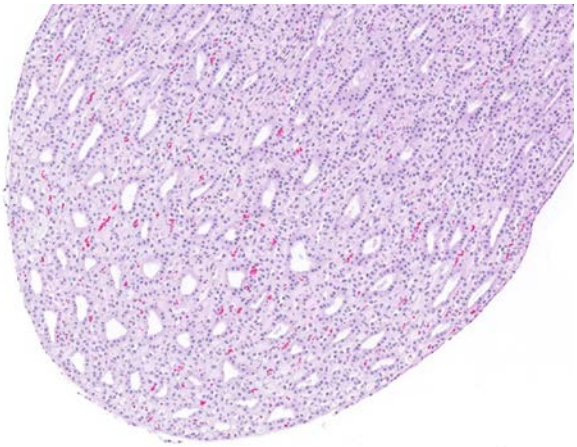


Figure 21. Magnified Area of the Papilla of the Vehicle Control Rat Shown in Figure 19 Demonstrating Normal Papilla (H&E)

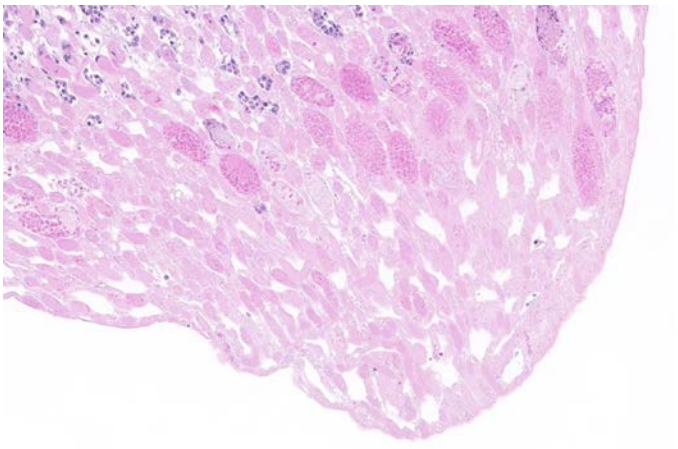


Figure 22. Magnified Area of the Papilla of the Treated Rat Shown in Figure 20 Demonstrating Necrosis of Papilla (H&E)

Necrosis is characterized by an area with the loss of differential staining, loss of cellular detail, and sloughing of epithelium.

Discussion

Due to widespread exposure to several of the per/polyfluorinated alkyl substances (PFAS), and few in vivo studies available for direct comparisons of toxicity across the class, a class comparison was conducted by NTP. A large amount of research has focused on perfluorooctane sulfonic acid (PFOS) and perfluorooctane octanoic acid (PFOA), as they are the most prominent PFAS in terms of exposure levels to date. Toxicity Study Report 97[104] and this report, Toxicity Study Report 96, detail results from 28-day studies evaluating the toxicity of seven straight-chained sulfonate and carboxylate PFAS of varying chain length. Wyeth-14,643 was included in the comparison studies as an indicator of potential peroxisome proliferator-activated receptor alpha (PPARα)-mediated toxicity. This section provides an overview of major endpoints of toxicity, a brief comparison across PFAS evaluated, and a discussion of potential mechanisms of toxicity.

These studies evaluated the plasma and liver (males only) concentrations after administration of perfluorobutane sulfonic acid (PFBS), perfluorohexane sulfonate potassium salt (PFHxSK), and PFOS for a comparison of toxicity. In general, the levels of each chemical were consistent with the literature on the kinetics of these particular PFAS, with the short-chain PFBS that has a shorter half-life displaying lower levels compared to the longer-chain (perfluorohexane sulfonate [PFHxS], PFOS) chemicals[31; 33; 34; 37]. In addition, the previously reported sex difference with PFHxS[31; 34] was apparent in this study, with female rats administered PFHxSK displaying much lower plasma concentrations. At the doses tested in this study, there was evidence of a nonlinear response occurring in males and females administered PFHxSK as evident in the steady decrease in plasma concentrations adjusted for dose. This is presumably due to saturation of protein or kidney transporter binding, resulting in a faster elimination rate. There was some evidence of saturation of kidney reuptake transporters or protein binding in males and females at the higher PFOS doses tested as plasma concentrations per dose administered were lower. However, this was not the case in PFBS males and females, which displayed an increase in plasma concentrations when adjusted for dose. The variation in nonlinear kinetics could be due to a multitude of factors (e.g., various protein or transporter binding constants) and highlights the individuality of kinetics across the PFAS. Overall, the levels of PFAS measured in these studies were generally orders of magnitude higher than measured in humans[9].

To evaluate accumulation in the liver, the ratios of liver/plasma concentrations were examined in male rats. In human blood, PFAS are generally localized in the plasma compartment compared to whole blood with ratios of 1.1 to 2.0 reported[105; 106], but this is not well understood in rodents. Liver/plasma ratios were highest in PFOS males, then PFHxSK males, and lowest in PFBS males. In general, the longer the chain length, the greater the distribution/accumulation observed within the liver. At the doses tested in these studies, the liver/plasma ratios decreased with increasing dose in PFOS males, suggesting a level of liver saturation or high plasma protein binding was reached.

The toxicity comparisons of a chemical could be evaluated from an exposure standpoint in several ways (e.g., comparing external dose versus internal dose metrics such as C_{max}, area under the curve [AUC], or final plasma measurements). In addition, each toxicity or effect may be better explained using a different exposure paradigm. An extensive evaluation of these possible exposure comparisons and response models is beyond the scope of this report, but a comparison

of findings based on external dose (mmol/kg/day) and plasma levels on day 29 (μM) allows for a qualitative summary of the findings and highlights the challenges in potency estimations. Liver and thyroid hormone findings in males and females were highlighted as they were generally present across all the chemicals (Figure 23, Figure 24, Figure 25, Figure 26, Figure 27, Figure 28, Figure 29, Figure 30, Figure 31, and Figure 32). Toxicity comparisons based on dose administered show PFOS to be the most potent and PFBS to be the least potent with PFHxSK closer to PFOS in potency than to PFBS. If plasma concentrations at day 29 are used for comparison, PFBS is generally the most potent (Figure 29, Figure 30, Figure 31, and Figure 32). However, it should be acknowledged that a significantly higher amount of PFBS had to be administered to the animals to attain these plasma levels and that AUC or C_{max} approaches may be better exposure metrics for comparison.

A major target organ for these three PFAS was the liver (Figure 23, Figure 24, and Figure 25), which is consistent with the PFAS class in general. In this study, all three alkyl sulfonates increased liver weights in both males and females. However, histologic lesions of the liver varied somewhat across these chemicals and between sexes. Hepatocyte cytoplasmic alteration was only observed in PFBS males and females and PFOS females. Hepatocyte hypertrophy was observed for each chemical in each sex except for female rats administered PFHxSK, which had no significant histologic effect in the liver (Table 15 and Figure 24). Although considerable amounts of PFHxSK were measured in female plasma (90 to 240 μM), and liver weight and *Cyp2b1* expression increased significantly, there was no evidence of PPARα activity via *Acox1* or *Cyp4a1* expression levels (Figure 24). PFOS females had a somewhat similar response of increased liver weights, *Cyp2b1* expression, and hepatocellular hypertrophy (Figure 25) in the presence of minimal increases in *Cyp4a1* expression. The lack of a PPARα response in females in the PFHxSK study may be a reason for the lack of hepatocellular hypertrophy response. Previous studies with PFHxS show hepatocellular hypertrophy at 3 mg/kg and higher in male rats[47] similar to what was observed in this study of PFHxSK. In a 90-day PFBS study, doses up to 600 mg/kg were administered once per day and hepatocyte hypertrophy was not reported[45]. It is unclear if the lack of hepatocyte hypertrophy in the PFBS 90-day study versus the observation in the current study is due to once-daily dosing versus the twice-daily dosing (e.g., C_{max} differences). Hepatocyte hypertrophy and cytoplasmic alteration were also observed with the PPARα agonist Wyeth-14,643. There was some evidence of necrosis in the highest dose group of PFBS males and females, but this was not observed in PFHxSK, PFOS, or Wyeth-14,643 groups at the doses administered.

All three alkyl sulfonates were inducers of *Cyp4a1* expression to a comparable magnitude in males (Figure 29) with PFBS being the least or most potent depending on the exposure metric. Within female rats, the induction of *Cyp4a1* was minimal with PFBS displaying the greatest magnitude of change, although it was still considerably lower than the male response. PFOS males displayed the greatest increase in *Cyp2b1* expression (Figure 29), whereas increases in *Cyp2b1* were observed in PFOS and PFBS females (Figure 30). The liver weight changes were of similar magnitude across the three chemicals, maxing at about 60% at the doses administered in males (Figure 29). There was no consistency in the magnitude of liver weight increases in females with a minimal response in PFHxSK compared to PFBS and PFOS (Figure 30). The lack of or minimal liver response in the PFHxSK females was very apparent (Figure 30) compared to the other chemicals and likely due to the lack of presumed activation of PPARα.

Perfluorinated compounds are known to mediate their effects through PPARα, CAR, pregnane X receptor (PXR), and other mechanisms[107; 108]. Hepatocyte hypertrophy observed with PFAS is likely due to the peroxisome proliferation. This is also supported by the elevated *Acox1* and *Cyp4a1* levels known to be inducible by PPARα agonists. Hepatocyte hypertrophy can also partially be mediated through CAR, because CAR-activated *Cyp2b1* is also elevated.

Increases in serum biomarkers associated with hepatobiliary injury were observed in the PFBS and PFOS groups. In general, bile acid concentrations had ≥ twofold increases in the higher-dose groups compared to concurrent vehicle controls. Increases of this magnitude are indicative of impaired bile flow or (intrahepatic) cholestasis, the causes of which include physical disruption of bile flow through the biliary system or perturbation of bile acid formation and excretion at the cellular level[109]. Direct bilirubin concentration, another marker of cholestasis, was minimally elevated in the high-dose groups of PFBS and PFOS. Alkaline phosphatase (ALP) activity was mildly increased, but is considered a poor marker of cholestasis in rats[110; 111]. Thus, while it may be increased due to cholestasis, limited studies have shown an association between the administration of PPARα agonists and mild increases in serum ALP activity when the only histologic effect observed was centrilobular hypertrophy[110; 112]. In general, there were mild increases in serum ALT, AST and SDH activities in the higher-dose groups of PFBS and PFOS. These enzymes are biomarkers of hepatocellular injury. Hepatocellular necrosis was not observed on histologic evaluation; however, hepatocellular injury resulting in enzyme leakage is associated with cholestasis[110]. In addition and similar to ALP activity, it has been shown that mild (≤1.5-fold) increases in hepatic and serum transaminases is associated with the administration of hepatic microsomal enzyme inducer compounds, including PPARα agonists[110; 112; 113]. (Note: hepatocellular necrosis was observed in the early death animals administered 1,000 mg/kg/day PFBS; however, there is no corresponding clinical chemistry.) Hepatobiliary injury biomarker changes similar to those observed in the current studies have been reported in the literature[20; 47; 114; 115].

There were consistent decreases in cholesterol concentrations in PFBS, PFHxSK, and PFOS males and to a much lesser extent in PFBS and PFOS females (high dose only). The magnitude of the effect was greatest in PFBS males and least in PFHxSK males. Similarly, a decrease in triglyceride concentrations was observed in PFBS, PFHxSK, and PFOS males and PFOS females. These changes are consistent with the known effects of PPARα activation on lipid metabolism, which include increases in peroxisomal fatty acid β-oxidation and effects on lipid transport. Studies have shown that administration of PPARα agonists, including PFAS, to rats results in lowered circulating triglyceride and cholesterol concentrations[47; 114-117]. CAR is also an important regulator of cholesterol homeostasis and its activation may also be related to the observed lipid alterations[118]. In addition, thyroid hormone triiodothyronine (T3) directly influences activities of critical enzymes in lipolytic pathways, and hypothyroidism in rats results in decreased blood triglyceride concentrations[119].

Globulin concentration was decreased in males administered all three perfluoroalkyl sulfonates. In most cases, the decrease in the globulin concentration resulted in an increase in the albumin/globulin ratio. The globulin fraction of serum total protein consists of the α-, β-, and γ-globulins. Alpha- and β-globulins are produced by hepatocytes and the γ-globulins (immunoglobulins) by B-lymphocytes. It is not known whether these chemicals are causing perturbation of the hepatic or lymphocytic production/metabolism of these proteins.

In addition, changes in thyroid hormone concentrations were observed across these three perfluoroalkyl sulfonates (Figure 26, Figure 27, and Figure 28). Total thyroxine (T4), free T4, and total T3 largely decreased in a dose-response manner. In general, the magnitude of the effect was stronger in PFBS and PFOS rats compared to the PFHxSK rats (Figure 31 and Figure 32). As the thyroid hormone levels decreased in a dose-response manner, thyroid stimulating hormone (TSH) concentration was not consistently increased across the chemicals or sexes in response to the lower thyroid hormone levels, nor were there any histopathologic changes in the thyroid gland (hyperplasia/hypertrophy). A study of PFHxS exposure reported follicular epithelial hypertrophy/hyperplasia presumably in response to lower thyroid hormones as thyroid hormones were not measured[47]. Several other studies[20; 52; 53; 120] of PFOS reported a similar pattern of decreased thyroid hormones without an increase in TSH. Production and release of thyroid hormones is regulated by thyroid-releasing hormone (hypothalamus) and TSH (pituitary gland) in what is referred to as the hypothalamic-pituitary-thyroid axis where decreases in circulating levels of free T4 and free T3 result in a compensatory increase in TSH. Archetypal xenobiotics that disrupt this axis in rats, causing decreases in thyroid hormones (either as a direct or indirect thyroid effect) with compensatory increases in TSH and thyroid gland follicular cell hyperplasia include sulfonamides, FD&C Red No. 3, and phenobarbital[121].

The reason for a lack of TSH response in the face of substantially low thyroid hormone concentrations in these sulfonate studies is not clear and not consistent with a disruption in the hypothalamic-pituitary-thyroid axis. It has been shown that PFAS can bind to proteins including albumin and transthyretin (previously prealbumin), which are transport proteins for thyroid hormones. Several PFOS studies (rat and monkey) have shown low free T4 levels as measured by analog radioimmunoassays (RIA) (the method used in the present studies), but no change in free T4 levels when measured by equilibrium dialysis followed by RIA (ED-RIA)[20; 51; 120; 122]. ED-RIA is considered the reference method for the determination of free T4 and is the standard by which other methods are compared. These findings are consistent with PFOS competing with free T4 for binding to serum proteins, creating a negative bias in the (competitive-binding) analog RIA method. A study evaluating the competitive binding of various PFAS to transthyretin was done by Weiss et al.[123] and confirmed the ability of these sulfonates to bind to transthyretin. Nevertheless, decreases in total T4 and T3 were found in the aforementioned studies and in the current studies. It is plausible that the decreases in total T4 and T3 are related to activation of PPARα and CAR resulting in an increase in thyroxine-UDP glucuronosyltransferase and accelerated degradation of thyroxine by the liver. It is noteworthy that PFHxSK had a lower response in CAR activity with a lower effect in thyroid hormones. While the total T4 was decreased, the lack of a TSH response and the ED-RIA findings of an unaffected free T4 from exposure to PFOS by several researchers does not support a state of classical hypothyroidism and is more similar to the profile of non-thyroidal illness syndrome (a.k.a., euthyroid sick syndrome)[124]. Other researchers have also concluded, in accordance with the thyroid-related results of their studies, that the administration of PFAS (PFDA, PFOS) does not cause a classical hypothyroid state[114; 120; 125].

Other sites of toxicity were the nose, forestomach, and kidney. Olfactory epithelium degeneration, hyperplasia, and inflammation were observed in PFBS males and females and PFHxSK females. Olfactory epithelium necrosis was also observed in males and females in the highest-dose group of PFBS, in which mortality was observed. In addition, forestomach epithelium hyperplasia was observed in PFBS males at the highest dose administered. These

nasal effects have not been observed in other PFHxSK studies, but nasal and stomach lesions were observed in male and female Sprague Dawley rats after a 90-day exposure[45], which the authors suggested could have been due to irritation from an oral gavage route of exposure. The pattern of nasal pathology in these studies was not indicative of gavage-related reflux by rats described by Damsch et al.[126]. Because olfactory epithelium can metabolize xenobiotics[127], the changes could be a direct effect of administration of PFAS. However, it is not possible to identify the underlying mechanism with the available data. None of the above lesions were observed in the Wyeth-14,643 positive controls suggesting that the mechanisms for these toxicities were not related to PPARα activation. Papilla necrosis of the kidney was also observed in the high-dose groups of PFBS males and females. A similar lesion was observed in the 90-day exposure study[45]. Papillary necrosis in the kidney has been reported to occur with numerous therapeutic agents and chemicals. However, the mechanism is still not completely understood[128-130].

Hematology parameters were altered for some of these chemicals. There were significant decreases in reticulocytes in PFBS males and females, PFHxSK males, and PFOS males and females. In addition, the hematocrit, hemoglobin concentration, and erythrocyte and reticulocyte numbers were all decreased in the PFBS males. These findings are consistent with the observed hypocellularity within the bone marrow of PFOS and PFBS males and females and the decreased extramedullary hematopoiesis in the spleen of PFOS males and females. The combination of these findings suggests a suppression in erythropoiesis. The suppression may be a direct effect or may be secondary to the stress of treatment (i.e., so-called anemia of chronic stress/disease). In addition, low thyroid hormones can cause decreases in the erythron[131]. Because rat erythrocytes have a circulating life span of approximately 60 days and the current studies were 28 days in duration, the hypocellularity in the bone marrow might not have been fully reflected in the circulating total red blood cell mass (i.e., erythrocyte count). Decreases in the erythron have also been observed in other studies with PFBS[45] and PFHxS[47], but not with PFOS. None of the above lesions were observed in the Wyeth-14,643 positive controls suggesting that the mechanisms for these toxicities were not related to PPARα activation.

PFBS and PFOS were negative in bacterial mutagenicity tests (PFHxSK and Wyeth-14,643 were not tested), and no indication of chromosomal damage, as measured in an erythrocyte micronucleus assay, was observed in male or female rats exposed to PFBS, PFHxSK, PFOS, and Wyeth-14,643. The bone marrow clearly was a target for these PFAS, as all three reduced the %PCEs in male rats as did PFBS and PFOS in female rats; however, %PCEs were not affected by PFHxSK in female rats. The cytotoxic effects of the three PFAS on PCEs in the micronucleus assay were very similar to observations in clinical chemistry tests.

In PFBS males and females, there were increases in thymic atrophy in the 1,000 mg/kg/day groups, which corresponded with a decrease in thymus weight in 1,000 mg/kg/day females. There was also a decrease in thymus weight in the female 500 mg/kg/day group. This change is most likely associated with stress, as thymic atrophy is one of the commonly recognized stress responses in toxicity studies[132]. Globulin levels decreased in PFBS males, which corresponded to increases in albumin/globulin ratios. These effects were not observed in a 90-day study of PFBS in male and female Sprague Dawley rats[45], in which 600 mg/kg was the highest dose administered. It may be the case that this an effect occurring at very high doses at which mortality occurred in this study.

The studies reported here and in Toxicity Study Report 97[104] provide data for a comparison of several members of the PFAS class. The liver and thyroid hormones were common targets for PFBS, PFHxSK, and PFOS, and similar effects were observed with the PFAS carboxylates[104]. In addition, findings of bone marrow hypocellularity, lesions of the forestomach, and lesions within the nose were present in several of the carboxylates studied[104]. Significant decreases in free T4, total T4, and total T3 levels occurred at the lowest doses tested for several of the PFAS except for PFHxSK females. In addition, increases in PPARα and CAR-related genes occurred at the lowest doses tested for PFBS and PFOS. In some cases, this corresponded with increased liver weights at the lowest dose tested. With the inclusion of the Wyeth-14,643 compound as a PPARα agonist, it was evident that several of the non-liver responses did not appear to be mediated via PPARα as they did not occur in rats administered Wyeth-14,643. Further comparison of these chemicals will require a more in-depth evaluation of the exposure between chemicals that could include AUC or C_{max} evaluations. The 28-day studies discussed in this report help to provide exposure and mechanistic context to facilitate extrapolation of findings.

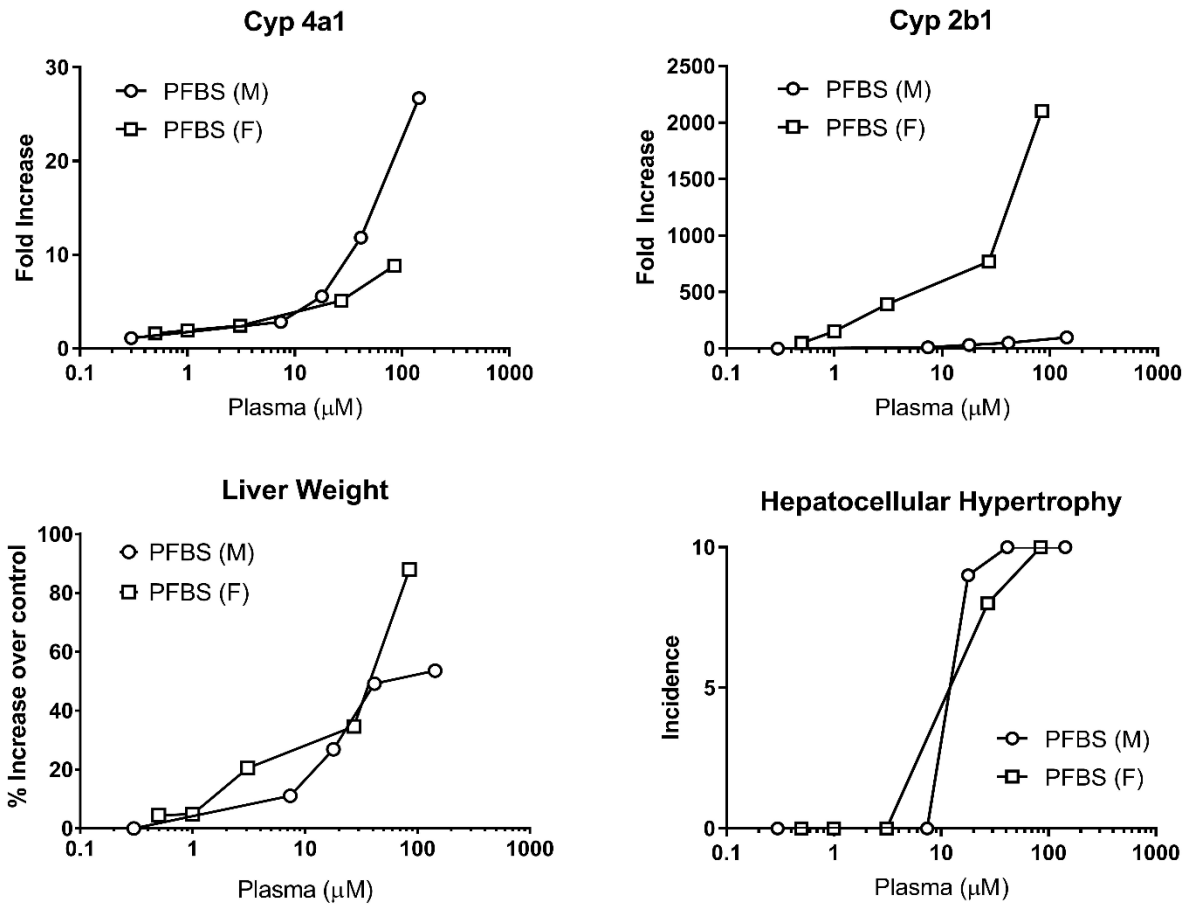


Figure 23. Comparison of Magnitude of Change for Select Liver Endpoints in Sprague Dawley Rats Administered Perfluorobutane Sulfonic Acid by Gavage for 28 Days

PFBS = perfluorobutane sulfonic acid.

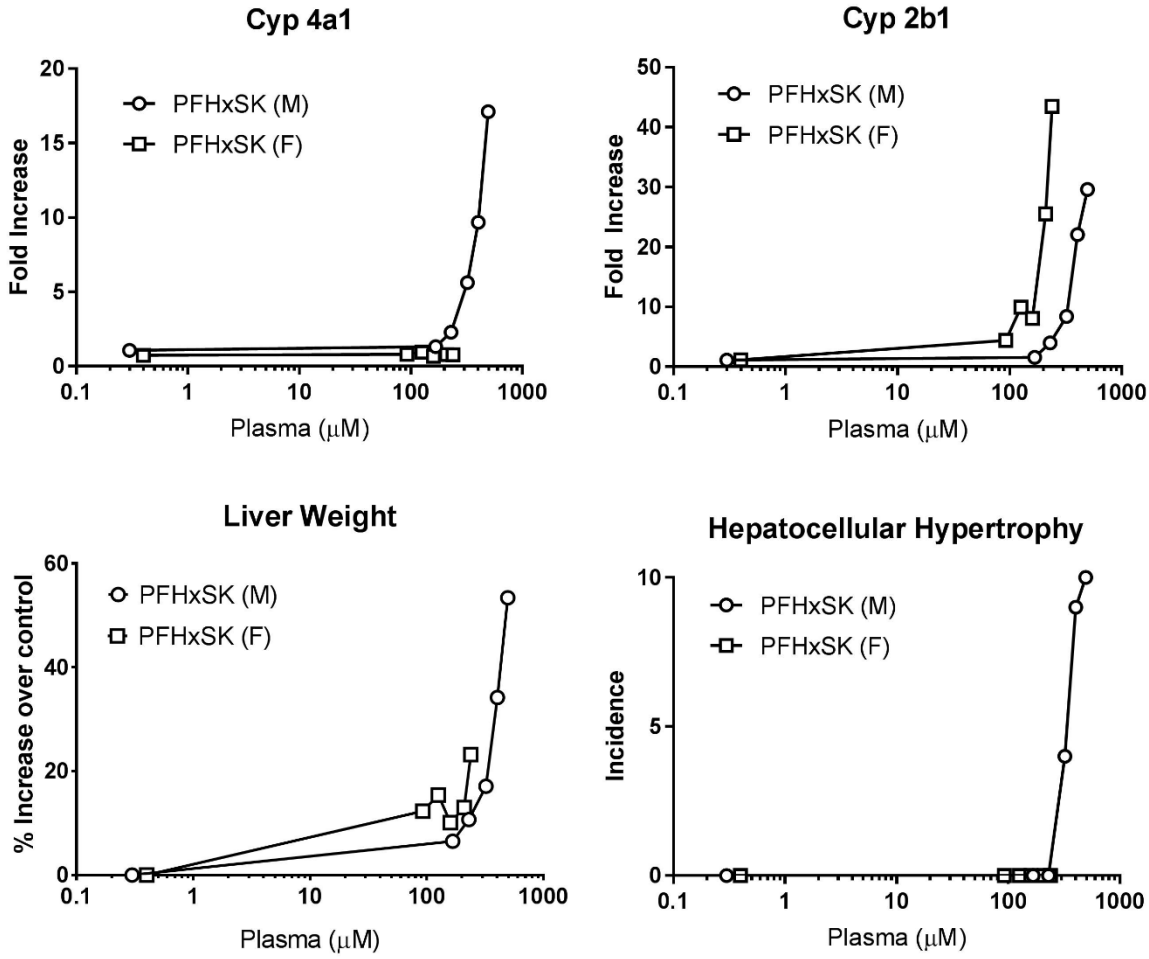


Figure 24. Comparison of Magnitude of Change for Select Liver Endpoints in Sprague Dawley Rats Administered Perfluorohexane Sulfonate Potassium Salt by Gavage for 28 Days

PFHxSK = perfluorohexane sulfonate potassium salt.

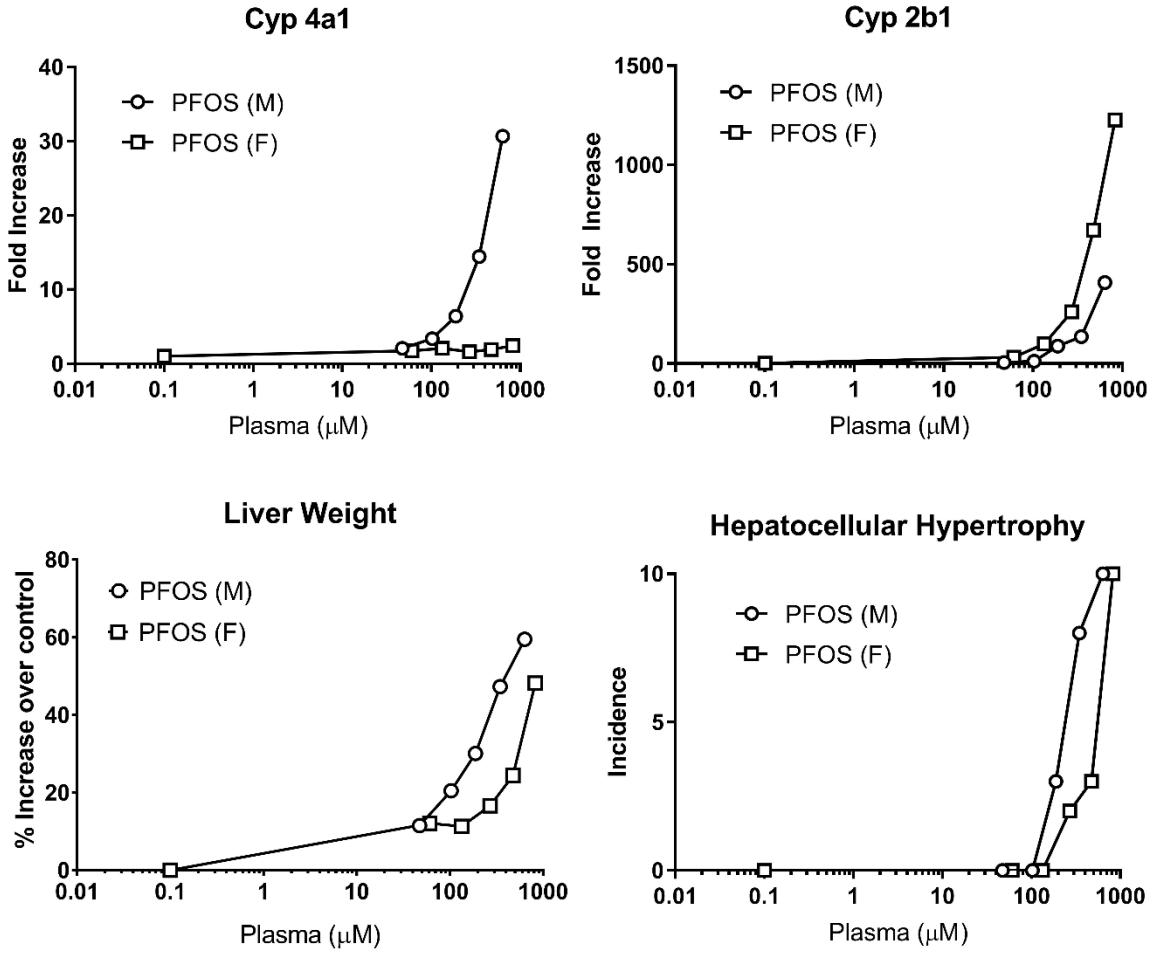


Figure 25. Comparison of Magnitude of Change for Select Liver Endpoints in Sprague Dawley Rats Administered Perfluorooctane Sulfonic Acid by Gavage for 28 Days

PFOS = perfluorooctane sulfonic acid.

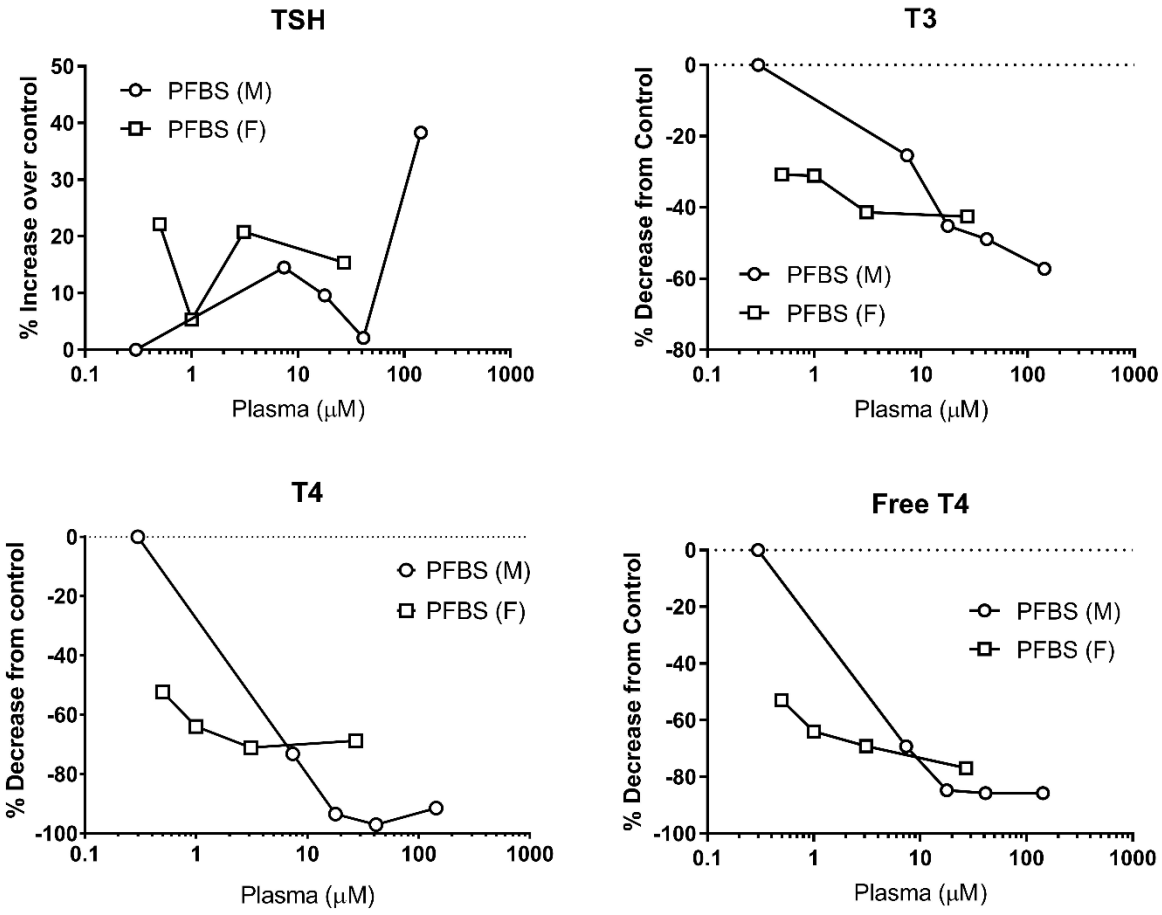


Figure 26. Comparison of Magnitude of Change for Thyroid Hormone Endpoints in Sprague Dawley Rats Administered Perfluorobutane Sulfonic Acid by Gavage for 28 Days

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine; PFBS = perfluorobutane sulfonic acid.

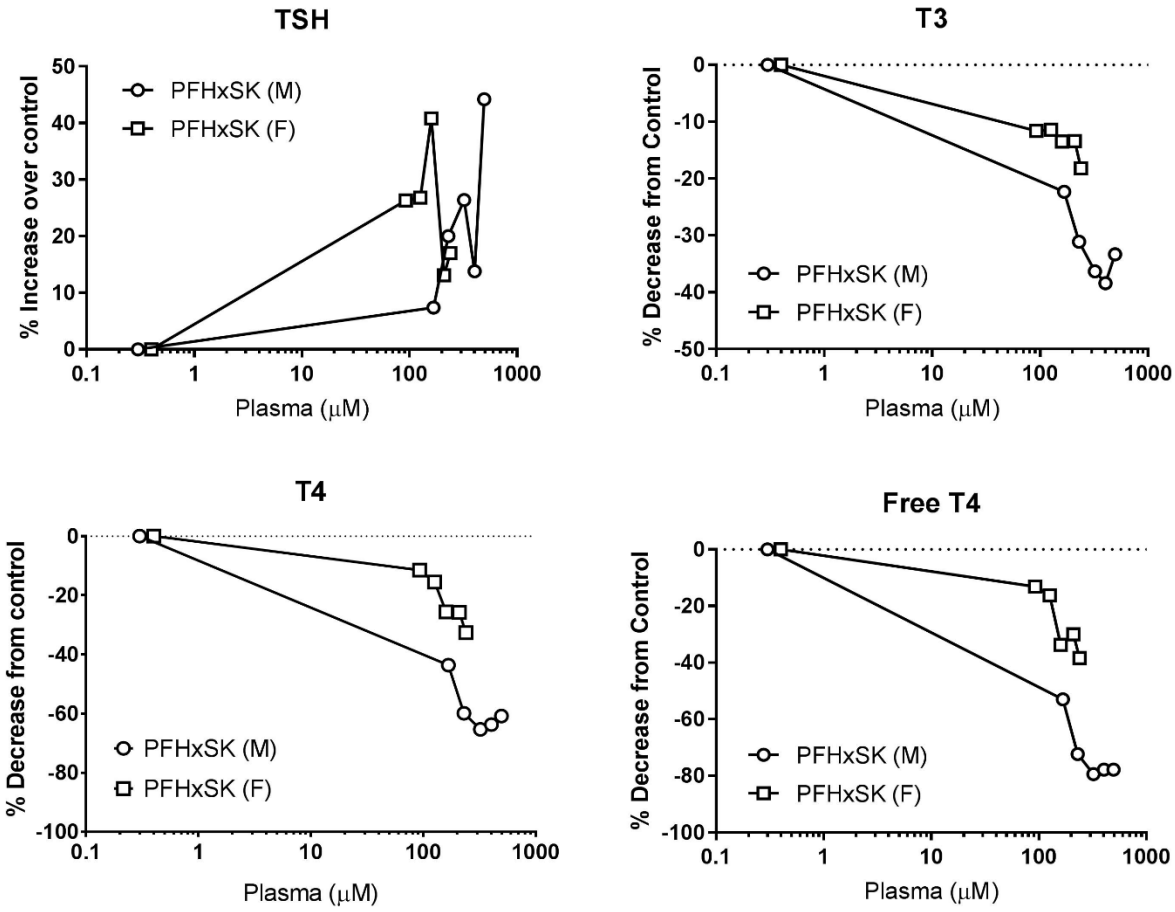


Figure 27. Comparison of Magnitude of Change for Thyroid Hormone Endpoints in Sprague Dawley Rats Administered Perfluorohexane Sulfonate Potassium Salt by Gavage for 28 Days

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine; PFHxSK = perfluorohexane sulfonate potassium salt.

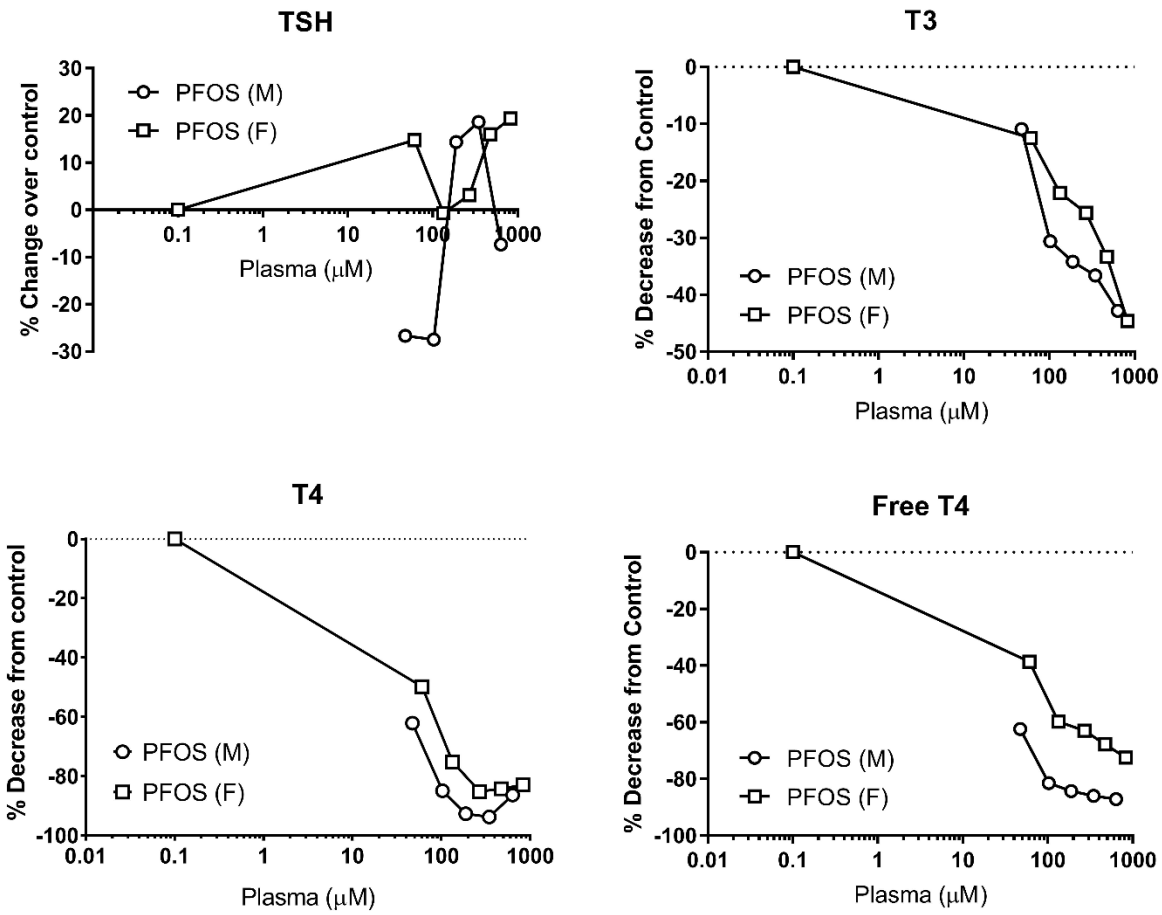


Figure 28. Comparison of Magnitude of Change for Thyroid Hormone Endpoints in Sprague Dawley Rats Administered Perfluorooctane Sulfonic Acid by Gavage for 28 Days

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine; PFOS = perfluorooctane sulfonic acid.

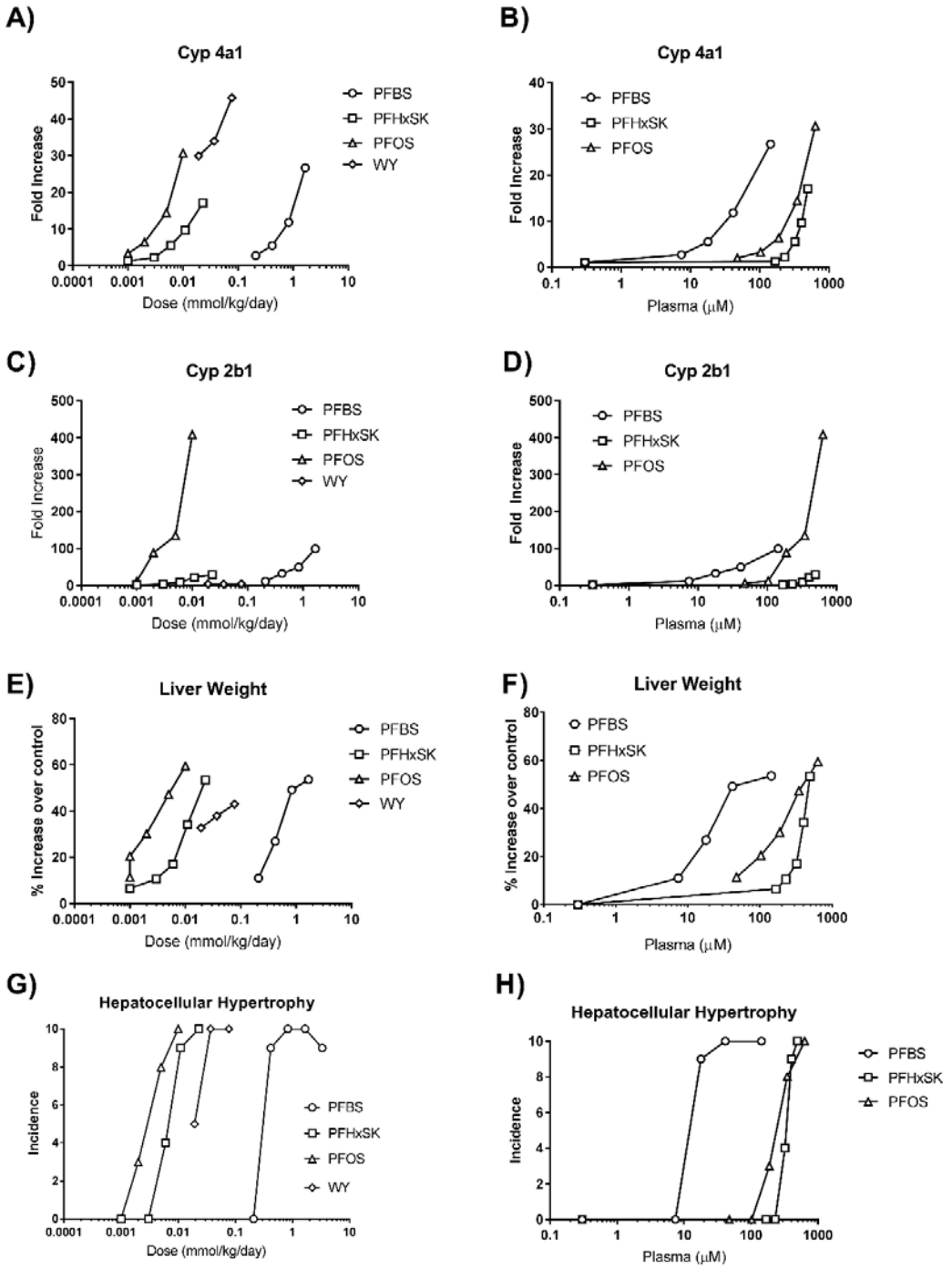


Figure 29. Average Magnitude of Change for *Cyp4a1* Expression (A, B), *Cyp2b1* Expression (C, D), Liver Weight Increase (E, F), and Hepatocellular Hypertrophy (G, H) in Male Sprague Dawley Rats Administered Perfluorobutane Sulfonic Acid, Perfluorohexane Sulfonate Potassium Salt, or Perfluorooctane Sulfonic Acid

PFBS = perfluorobutane sulfonic acid; PFHxSK = perfluorohexane sulfonate potassium salt; PFOS = perfluorooctane sulfonic acid; WY = Wyeth-14,643.
Comparisons are made on a dose administered (mol/kg/day) basis (A, C, E, G) and plasma level (μM) basis (B, D, F, H).
Wyeth-14,643 (PPARα agonist) used for comparison on a dose administered (mol/kg/day) basis (A, C, E, G).

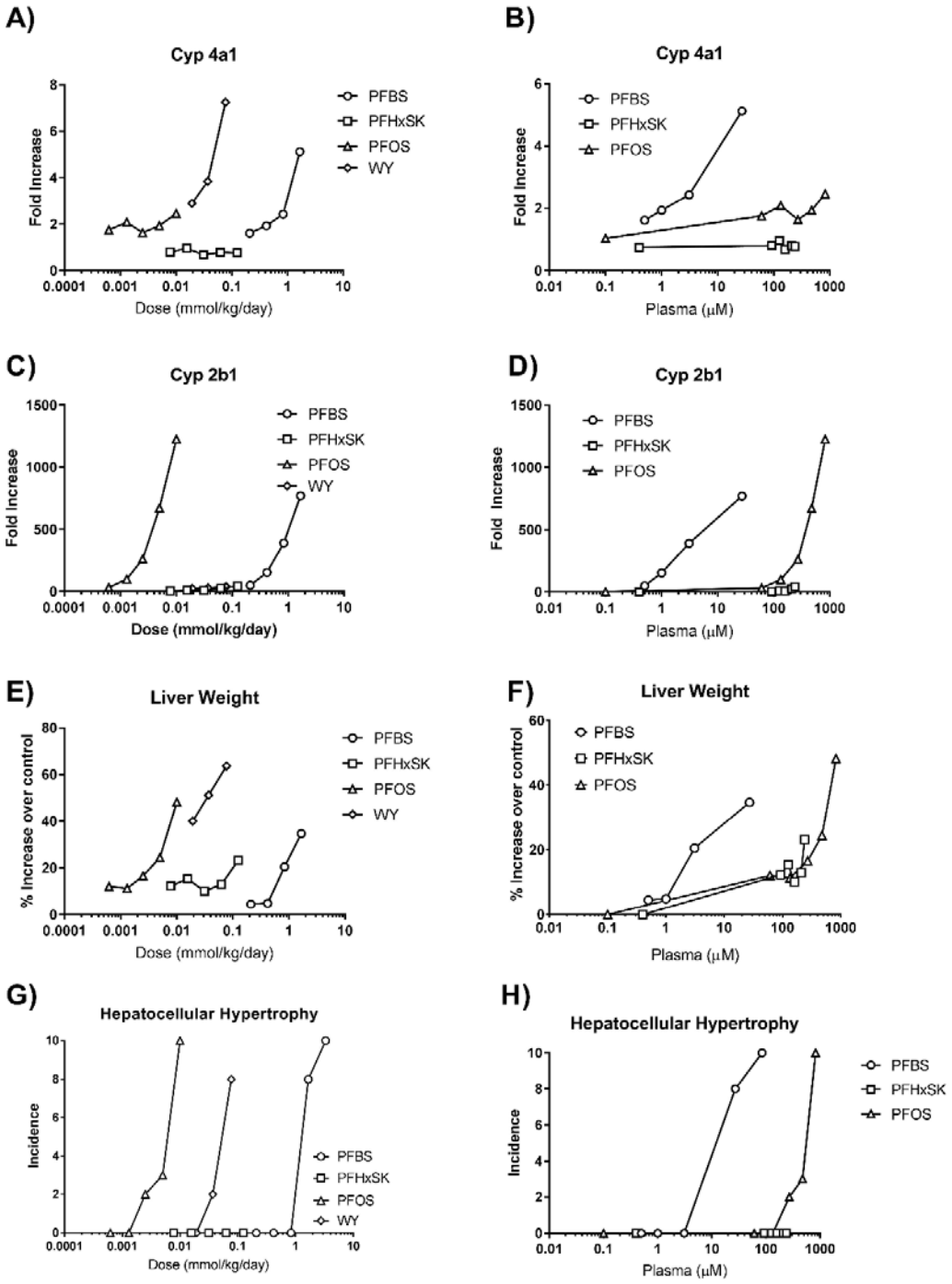


Figure 30. Average Magnitude of Change for *Cyp4a1* Expression (A, B), *Cyp2b1* Expression (C, D), Liver Weight Increase (E, F), and Hepatocellular Hypertrophy (G, H) in Female Sprague Dawley Rats Administered Perfluorobutane Sulfonic Acid, Perfluorohexane Sulfonate Potassium Salt, or Perfluorooctane Sulfonic Acid

PFBS = perfluorobutane sulfonic acid; PFHxSK = perfluorohexane sulfonate potassium salt; PFOS = perfluorooctane sulfonic acid; WY = Wyeth-14,643.
Comparisons are made on a dose administered (mol/kg/day) basis (A, C, E, G) and plasma level (μM) basis (B, D, F, H).
Wyeth-14,643 (PPARα agonist) used for comparison on a dose administered (mol/kg/day) basis (A, C, E, G).

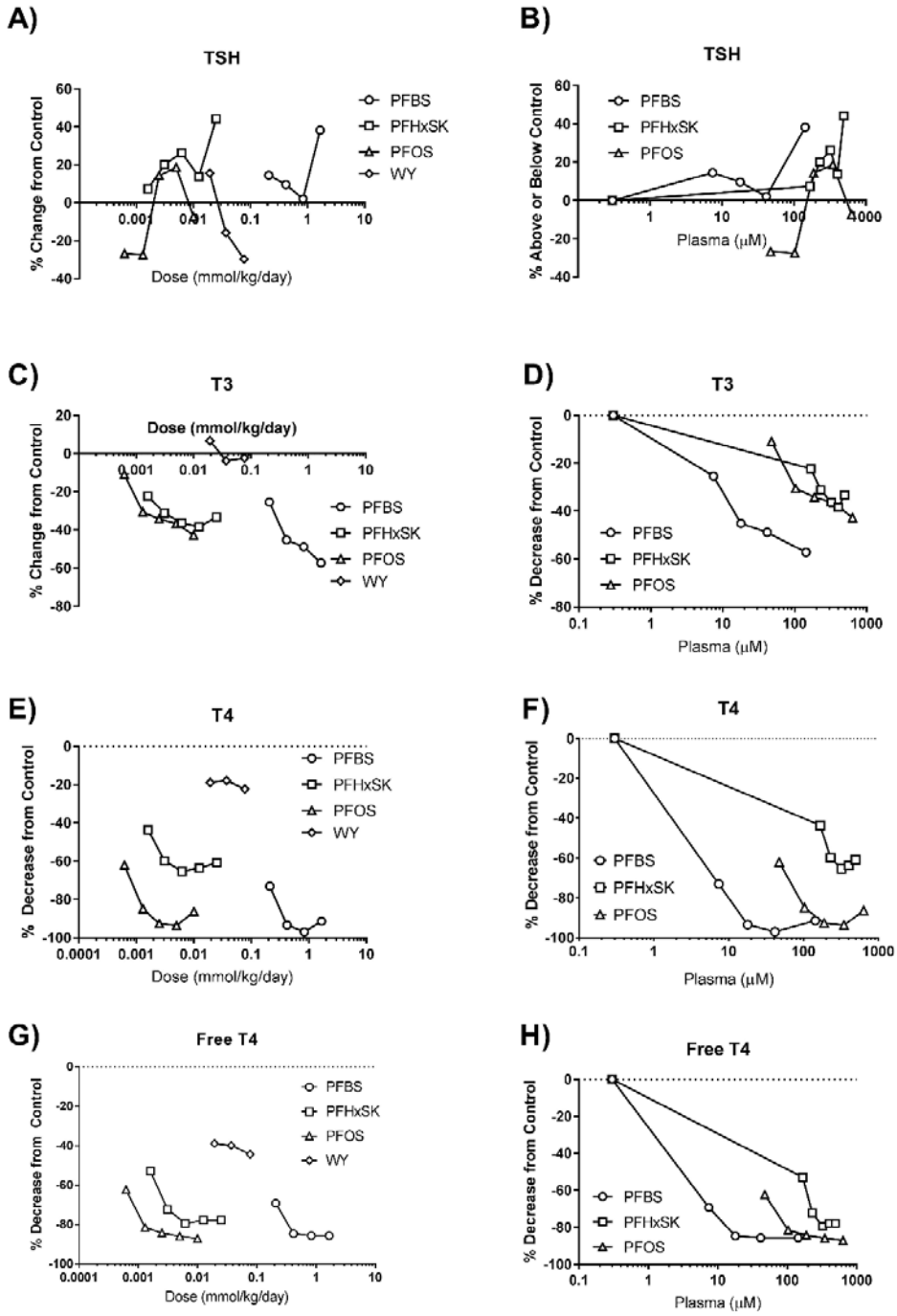


Figure 31. Comparison of Magnitude of Change for Effects on Serum Levels of Thyroid Stimulating Hormone (A, B), Triiodothyronine (C, D), Thyroxine (E, F), and Free Thyroxine (G, H) for Perfluorobutane Sulfonic Acid, Perfluorohexane Sulfonate Potassium Salt, or Perfluorooctane Sulfonic Acid in Male Rats

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine;
PFBS = perfluorobutane sulfonic acid; PFHxSK = perfluorohexane sulfonate potassium salt; PFOS = perfluorooctane sulfonic acid; WY = Wyeth-14,643.
Comparisons are made on a dose administered (mol/kg/day) basis (A, C, E, G) and plasma level (μM) basis (B, D, F, H).
Wyeth-14,643 (PPARα agonist) used for comparison on a dose administered (mol/kg/day) basis (A, C, E, G).

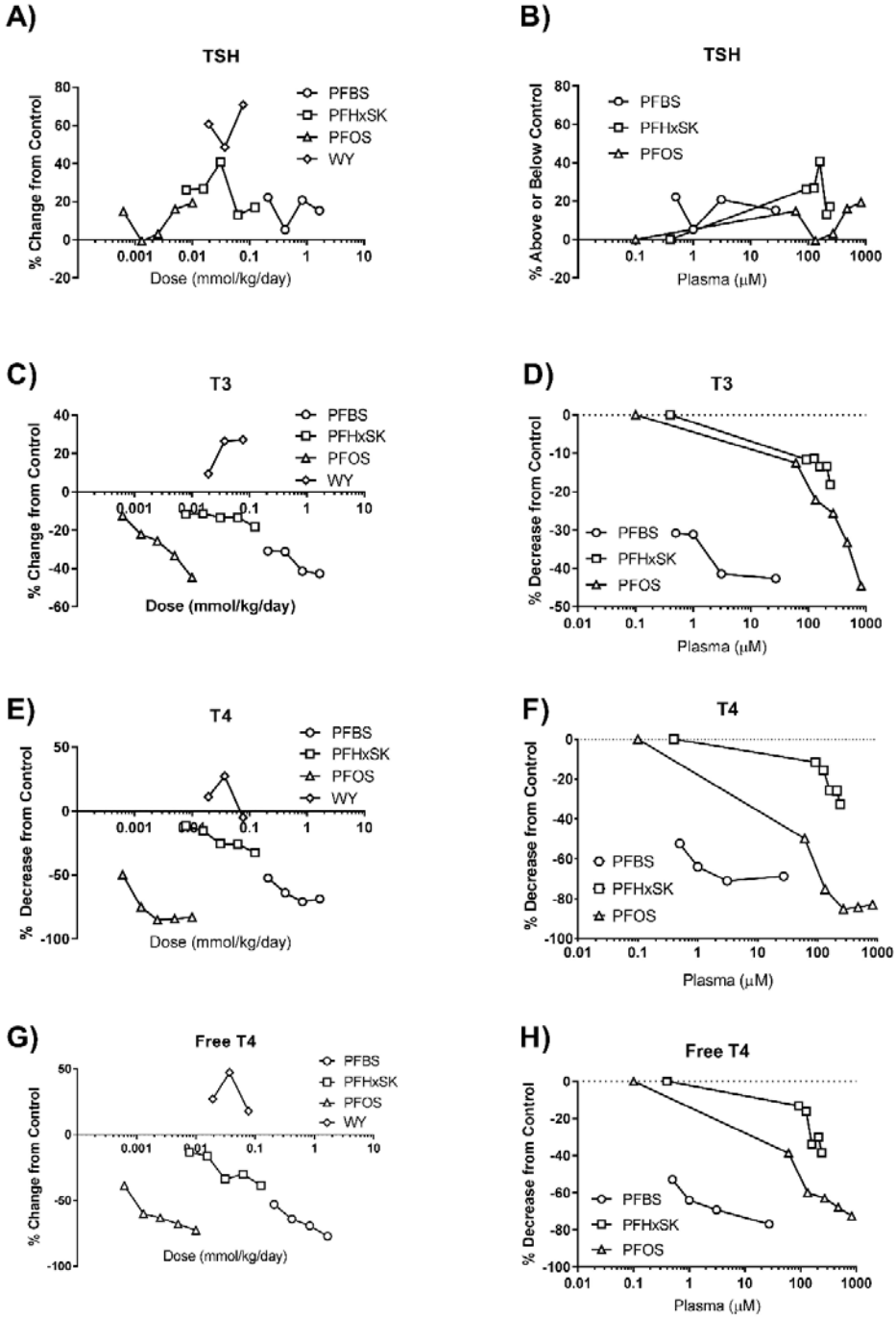


Figure 32. Comparison of Magnitude of Change for Effects on Serum Levels of Thyroid Stimulating Hormone (A, B), Triiodothyronine (C, D), Thyroxine (E, F), and Free Thyroxine (G, H) for Perfluorobutane Sulfonic Acid, Perfluorohexane Sulfonate Potassium Salt, or Perfluorooctane Sulfonic Acid in Female Rats

TSH = thyroid stimulating hormone; T3 = triiodothyronine; T4 = total thyroxine; free T4 = free thyroxine;
PFBS = perfluorobutane sulfonic acid; PFHxSK = perfluorohexane sulfonate potassium salt; PFOS = perfluorooctane sulfonic acid; WY = Wyeth-14,643.
Comparisons are made on a dose administered (mol/kg/day) basis (A, C, E, G) and plasma level (μM) basis (B, D, F, H)
Wyeth-14,643 (PPARα agonist) used for comparison on a dose administered (mol/kg/day) basis (A, C, E, G).

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Appendix A. Reproductive Tissue Evaluations and Estrous Cycle Characterization

Tables

Figures

Table A-1. Summary of Reproductive Tissue Evaluations for Male Rats in the 28-day Gavage Study of Perfluorobutane Sulfonic Acid[a]

	Vehicle Control	62.6 mg/kg/day	125 mg/kg/day	250 mg/kg/day	500 mg/kg/day
n	10	10	10	10	10
Weights (g)					
Necropsy Body Wt.	342 ± 7	335 ± 8	349 ± 9	348 ± 9	317 ± 6
L. Cauda Epididymis	0.195 ± 0.005	–	0.181 ± 0.007	0.194 ± 0.007	0.195 ± 0.005
L. Epididymis	0.531 ± 0.008	–	0.517 ± 0.012	0.532 ± 0.014	0.532 ± 0.011
L. Testis	1.786 ± 0.026	–	1.818 ± 0.041	1.828 ± 0.053	1.891 ± 0.033
Spermatid Measurements					
Spermatid Heads (10^6/testis)	300.5 ± 12.0	–	295.4 ± 10.9	269.9 ± 12.9	287.9 ± 10.2
Spermatid Heads (10^6/g testis)	168.3 ± 6.5	–	163.0 ± 5.9	147.4 ± 4.6	152.3 ± 5.0
Epididymal Spermatozoal Measurements					
Sperm Motility (%)	87.9 ± 0.2	–	87.0 ± 0.6	88.0 ± 0.3	86.8 ± 0.5
Sperm (10^6/Cauda Epididymis)	132.9 ± 12.0	–	113.0 ± 8.2	115.6 ± 4.7	112.3 ± 7.3
Sperm (10^6/g Cauda Epididymis)	677 ± 53	–	632 ± 51	599 ± 27	575 ± 30
Testosterone (ng/mL)[b]	3.241 ± 0.508[c]	3.102 ± 0.767	2.537 ± 0.535	4.111 ± 0.582[c]	3.724 ± 1.293[c]

[a]Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Williams' or Dunnett's test (weights) or Shirley's or Dunn's test (spermatid, epididymal spermatozoal, and testosterone measurements). One-half the dose was administered twice daily.
[b]Reproductive parameters were not evaluated in 62.6 mg/kg/day males, and testosterone was not measured in 1,000 m/kg/day males.
[c]n = 9.

Table A-2. Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Rats Administered Perfluorobutane Sulfonic Acid by Gavage for 28 Days

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	<0.001	
Overall Tests	125 mg/kg/day vs. vehicle controls	0.449	–
Overall Tests	250 mg/kg/day vs. vehicle controls	<0.001	–
Overall Tests	500 mg/kg/day vs. vehicle controls	<0.001	–
Extended Estrus	Overall	<0.001	
Extended Estrus	125 mg/kg/day vs. vehicle controls	0.15	–
Extended Estrus	250 mg/kg/day vs. vehicle controls	0.058	N
Extended Estrus	500 mg/kg/day vs. vehicle controls	<0.001	N
Extended Diestrus	Overall	<0.001	
Extended Diestrus	125 mg/kg/day vs. vehicle controls	0.063	–
Extended Diestrus	250 mg/kg/day vs. vehicle controls	<0.001	–
Extended Diestrus	500 mg/kg/day vs. vehicle controls	<0.001	–
Extended Metestrus	Overall	1	
Extended Metestrus	125 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	250 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	500 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	Overall	1	
Extended Proestrus	125 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	250 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	500 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	Overall	0.999	
Skipped Estrus	125 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	250 mg/kg/day vs. vehicle controls	0.931	–
Skipped Estrus	500 mg/kg/day vs. vehicle controls	0.92	–
Skipped Diestrus	Overall	0.464	
Skipped Diestrus	125 mg/kg/day vs. vehicle controls	0.321	N
Skipped Diestrus	250 mg/kg/day vs. vehicle controls	0.343	N
Skipped Diestrus	500 mg/kg/day vs. vehicle controls	0.411	N
Summary of Significant Groups			
Overall Tests	250 mg/kg/day vs. vehicle controls	<0.001	–
Overall Tests	500 mg/kg/day vs. vehicle controls	<0.001	–
Extended Estrus	500 mg/kg/day vs. vehicle controls	<0.001	N
Extended Diestrus	250 mg/kg/day vs. vehicle controls	<0.001	–
Extended Diestrus	500 mg/kg/day vs. vehicle controls	<0.001	–

[a]N indicates that the dose group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dose group had more departures from normal cycling than did the vehicle control group. The Overall Tests consider all six types of departures from normal cycling. The overall comparison tests for equal transition probabilities across all dose groups. One-half the dose was administered twice daily.

Table A-3. Summary of Reproductive Tissue Evaluations for Male Rats in the 28-day Gavage Study of Perfluorohexane Sulfonate Potassium Salt[a]

	Vehicle Control	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day	10 mg/kg/day
n	10	10	10	10	10	10
Weights (g)						
Necropsy Body Wt.	336 ± 7	346 ± 8	342 ± 7	343 ± 6	341 ± 5	335 ± 6
L. Cauda Epididymis	0.198 ± 0.008	–	–	0.195 ± 0.005	0.195 ± 0.007	0.190 ± 0.004
L. Epididymis	0.548 ± 0.011	–	–	0.532 ± 0.010	0.538 ± 0.017	0.537 ± 0.008
L. Testis	1.842 ± 0.051	–	–	1.879 ± 0.056	1.846 ± 0.062	1.850 ± 0.019
Spermatid Measurements						
Spermatid Heads (10^6/Testis)	212.5 ± 7.1	–	–	220.0 ± 8.8	215.1 ± 14.0	213.3 ± 7.3
Spermatid Heads (10^6/g Testis)	115.8 ± 4.2	–	–	118.0 ± 5.8	116.7 ± 6.7	115.6 ± 4.6
Epididymal Spermatozoal Measurements						
Sperm Motility (%)	83.4 ± 2.3	–	–	85.9 ± 0.7	85.0 ± 0.8	85.3 ± 0.7
Sperm (10^6/Cauda Epididymis)	131.4 ± 13.2	–	–	144.0 ± 8.7	125.7 ± 9.7	128.6 ± 9.1
Sperm (10^6/g Cauda Epididymis)	653 ± 51	–	–	735 ± 35	651 ± 54	675 ± 42
Testosterone (ng/mL)[b]	4.595 ± 0.680	7.739 ± 1.440	6.500 ± 1.660	8.111 ± 1.747	5.875 ± 1.141	7.142 ± 1.267

[a]Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Williams' or Dunnett's test (weights) or Shirley's or Dunn's test (spermatid, epididymal spermatozoal, and testosterone measurements).
[b]Reproductive parameters were not evaluated in 0.625 or 1.25 mg/kg/day males.

Table A-4. Estrous Cycle Characterization for Female Rats in the 28-day Gavage Study of Perfluorohexane Sulfonate Potassium Salt[a]

	Vehicle Control	12.5 mg/kg/day	25 mg/kg/day	50 mg/kg/day
n	10	10	9	10
Necropsy Body Wt. (g)	224 ± 3	227 ± 4	230 ± 3[b]	230 ± 3
Number of Estrous Cycles	2.0 ± 0.0	1.9 ± 0.18	1.8 ± 0.15	2.1 ± 0.28
Estrous Cycle Length (Days)	5.2 ± 0.21	5.5 ± 0.64	5.3 ± 0.60	4.5 ± 0.20
Estrous Stages (% of Cycle)				
Diestrus	50.6	52.5	56.9	55.6
Proestrus	8.1	11.9	6.3	8.1
Estrus	39.4	33.1	36.3	31.3
Metestrus	1.9	2.5	0.6	5.0

[a]Necropsy body weights, number of estrous cycles, and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunneett's test (body weight) or Dunn's test (estrous cycle number and length). Tests for equality of transition probability matrices among all groups and between the vehicle control group and each dosed group indicated that dosed females did not have extended estrus or diestrus.
[b]n = 10.

Table A-5. Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Rats Administered Perfluorohexane Sulfonate Potassium Salt by Gavage for 28 Days

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	<0.001	
Overall Tests	12.5 mg/kg/day vs. vehicle controls	0.004	N
Overall Tests	25 mg/kg/day vs. vehicle controls	<0.001	N
Overall Tests	50 mg/kg/day vs. vehicle controls	0.122	N
Extended Estrus	Overall	0.006	
Extended Estrus	12.5 mg/kg/day vs. vehicle controls	0.054	N
Extended Estrus	25 mg/kg/day vs. vehicle controls	0.038	N
Extended Estrus	50 mg/kg/day vs. vehicle controls	0.054	N
Extended Diestrus	Overall	0.002	
Extended Diestrus	12.5 mg/kg/day vs. vehicle controls	0.117	N
Extended Diestrus	25 mg/kg/day vs. vehicle controls	<0.001	N
Extended Diestrus	50 mg/kg/day vs. vehicle controls	0.644	–
Extended Metestrus	Overall	1	
Extended Metestrus	12.5 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	25 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	50 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	Overall	1	
Extended Proestrus	12.5 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	25 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	50 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	Overall	0.999	
Skipped Estrus	12.5 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	25 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	50 mg/kg/day vs. vehicle controls	0.87	–
Skipped Diestrus	Overall	1	
Skipped Diestrus	12.5 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	25 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	50 mg/kg/day vs. vehicle controls	1	–
Summary of Significant Groups			
Overall Tests	12.5 mg/kg/day vs. vehicle controls	0.004	N
Overall Tests	25 mg/kg/day vs. vehicle controls	<0.001	N
Extended Estrus	25 mg/kg/day vs. vehicle controls	0.038	N
Extended Diestrus	25 mg/kg/day vs. vehicle controls	<0.001	N

[a]N indicates that the dose group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dose group had more departures from normal cycling than did the vehicle control group. The Overall Tests consider all six types of departures from normal cycling. The overall comparison tests for equal transition probabilities across all dose groups.

Table A-6. Summary of Reproductive Tissue Evaluations for Male Rats in the 28-day Gavage Study of Perfluorooctane Sulfonic Acid[a]

	Vehicle Control	0.312 mg/kg/day	0.625 mg/kg/day	1.25 mg/kg/day	2.5 mg/kg/day	5 mg/kg/day
n	10	10	10	10	10	10
Weights (g)						
Necropsy Body Wt.	337 ± 7	340 ± 5	338 ± 7	331 ± 4	333 ± 4	309 ± 4**
L. Cauda Epididymis	0.195 ± 0.007	–	–	0.182 ± 0.004	0.183 ± 0.006	0.195 ± 0.006
L. Epididymis	0.545 ± 0.015	–	–	0.520 ± 0.010	0.509 ± 0.008	0.524 ± 0.014
L. Testis	1.864 ± 0.025	–	–	1.746 ± 0.038	1.766 ± 0.037	1.848 ± 0.035
Spermatid Measurements						
Spermatid Heads (10^6/Testis)	267.0 ± 12.0	–	–	249.0 ± 10.2	262.5 ± 13.1	264.0 ± 14.4
Spermatid Heads (10^6/g Testis)	143.2 ± 6.4	–	–	142.7 ± 5.2	148.2 ± 5.6	142.4 ± 6.0
Epididymal Spermatozoal Measurements						
Sperm Motility (%)	87.5 ± 0.4	–	–	87.8 ± 0.3	87.3 ± 0.4	88.4 ± 0.4
Sperm (10^6/Cauda Epididymis)	114.0 ± 6.5	–	–	104.0 ± 6.9	107.5 ± 6.1	120.8 ± 8.6
Sperm (10^6/g Cauda Epididymis)	591 ± 37	–	–	572 ± 35	592 ± 38	622 ± 40
Testosterone (ng/mL)[b]	3.775 ± 0.754	5.812 ± 1.602	5.074 ± 1.718[c]	4.432 ± 1.721	5.576 ± 1.167	1.867 ± 1.367

**Significantly different ($p \leq 0.01$) from the vehicle control group by Williams' or Dunnett's test.
[a]Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Williams' or Dunnett's test (tissue weights) or Shirley's or Dunn's test (spermatid, epididymal spermatozoal, and testosterone measurements).
[b]Reproductive parameters were not evaluated in 0.312 or 0.625 mg/kg/day males.
[c]n = 9.

Table A-7. Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Rats Administered Perfluorooctane Sulfonic Acid by Gavage for 28 Days

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	<0.001	
Overall Tests	0.625 mg/kg/day vs. vehicle controls	<0.001	–
Overall Tests	1.25 mg/kg/day vs. vehicle controls	0.004	–
Overall Tests	2.5 mg/kg/day vs. vehicle controls	0.001	–
Extended Estrus	Overall	0.034	
Extended Estrus	0.625 mg/kg/day vs. vehicle controls	0.059	N
Extended Estrus	1.25 mg/kg/day vs. vehicle controls	0.097	–
Extended Estrus	2.5 mg/kg/day vs. vehicle controls	0.191	–
Extended Diestrus	Overall	<0.001	
Extended Diestrus	0.625 mg/kg/day vs. vehicle controls	0.001	–
Extended Diestrus	1.25 mg/kg/day vs. vehicle controls	0.028	–
Extended Diestrus	2.5 mg/kg/day vs. vehicle controls	<0.001	–
Extended Metestrus	Overall	0.917	
Extended Metestrus	0.625 mg/kg/day vs. vehicle controls	0.363	–
Extended Metestrus	1.25 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	2.5 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	Overall	1	
Extended Proestrus	0.625 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	1.25 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	2.5 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	Overall	0.012	
Skipped Estrus	0.625 mg/kg/day vs. vehicle controls	0.003	–
Skipped Estrus	1.25 mg/kg/day vs. vehicle controls	0.312	N
Skipped Estrus	2.5 mg/kg/day vs. vehicle controls	0.312	N
Skipped Diestrus	Overall	1	
Skipped Diestrus	0.625 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	1.25 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	2.5 mg/kg/day vs. vehicle controls	1	–
Summary of Significant Groups			
Overall Tests	0.625 mg/kg/day vs. vehicle controls	<0.001	–
Overall Tests	1.25 mg/kg/day vs. vehicle controls	0.004	–
Overall Tests	2.5 mg/kg/day vs. vehicle controls	0.001	–
Extended Diestrus	0.625 mg/kg/day vs. vehicle controls	0.001	–
Extended Diestrus	1.25 mg/kg/day vs. vehicle controls	0.028	–
Extended Diestrus	2.5 mg/kg/day vs. vehicle controls	<0.001	–
Skipped Estrus	0.625 mg/kg/day vs. vehicle controls	0.003	–

[a]N indicates that the dose group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dose group had more departures from normal cycling than did the vehicle control group. The Overall Tests consider all six types of departures from normal cycling. The overall comparison tests for equal transition probabilities across all dose groups.

Table A-8. Results of Vaginal Cytology Study Using the Transition Matrix Approach in Female Rats Administered Wyeth-14,643 by Gavage for 28 Days

Stage	Comparison	P Value	Trend[a]
Overall Tests	Overall	0.002	
Overall Tests	6.25 mg/kg/day vs. vehicle controls	0.085	–
Overall Tests	12.5 mg/kg/day vs. vehicle controls	<0.001	N
Overall Tests	25 mg/kg/day vs. vehicle controls	0.983	–
Extended Estrus	Overall	<0.001	
Extended Estrus	6.25 mg/kg/day vs. vehicle controls	0.032	N
Extended Estrus	12.5 mg/kg/day vs. vehicle controls	0.001	N
Extended Estrus	25 mg/kg/day vs. vehicle controls	0.017	N
Extended Diestrus	Overall	<0.001	
Extended Diestrus	6.25 mg/kg/day vs. vehicle controls	0.005	–
Extended Diestrus	12.5 mg/kg/day vs. vehicle controls	0.001	–
Extended Diestrus	25 mg/kg/day vs. vehicle controls	0.009	–
Extended Metestrus	Overall	1	
Extended Metestrus	6.25 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	12.5 mg/kg/day vs. vehicle controls	1	–
Extended Metestrus	25 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	Overall	1	
Extended Proestrus	6.25 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	12.5 mg/kg/day vs. vehicle controls	1	–
Extended Proestrus	25 mg/kg/day vs. vehicle controls	1	–
Skipped Estrus	Overall	0.572	
Skipped Estrus	6.25 mg/kg/day vs. vehicle controls	0.316	N
Skipped Estrus	12.5 mg/kg/day vs. vehicle controls	0.319	N
Skipped Estrus	25 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	Overall	1	
Skipped Diestrus	6.25 mg/kg/day vs. vehicle controls	1	–
Skipped Diestrus	12.5 mg/kg/day vs. vehicle controls	0.934	–
Skipped Diestrus	25 mg/kg/day vs. vehicle controls	1	–
Summary of Significant Groups			
Overall Tests	12.5 mg/kg/day vs. vehicle controls	<0.001	N
Extended Estrus	6.25 mg/kg/day vs. vehicle controls	0.032	N
Extended Estrus	12.5 mg/kg/day vs. vehicle controls	0.001	N
Extended Estrus	25 mg/kg/day vs. vehicle controls	0.017	N
Extended Diestrus	6.25 mg/kg/day vs. vehicle controls	0.005	–
Extended Diestrus	12.5 mg/kg/day vs. vehicle controls	0.001	–
Extended Diestrus	25 mg/kg/day vs. vehicle controls	0.009	–

[a]N indicates that the dose group had fewer departures from normal cycling than did the vehicle control group. Having no N indicates that the dose group had more departures from normal cycling than did the vehicle control group. The Overall Tests consider all six types of departures from normal cycling. The overall comparison tests for equal transition probabilities across all dose groups.

Dose (mg/kg /day)	Animal ID																												
0	211									D	P	E	E	D	D	D	P	E	D	D	D	E	E	E	D				
0	212										D	P	E	E	D	D	D	E	E	D	D	D	E	E	M	D			
0	213												D	E	D	D	P	E	D	D	D	E	E	E	D	P	E	D	
0	214											P	E	E	D	D	D	E	E	D	D	D	P	E	E	D	E		
0	215									D	E	M	D	D	D	D	P	E	D	D	E	E	D	D	I				
0	216						D	D	D	D	D	D	D	D	E	D	D	P	E	E	D	D								
0	217									D	D	E	D	D	D	D	P	E	D	D	P	E	E	D	D				
0	218									D	D	D	E	E	D	D	D	E	E	D	D	D	P	E	E				
0	219													E	E	D	P	E	M	E	E	D	D	D	E	E	D	D	D
0	220										D	D	E	D	D	D	D	E	E	D	D	P	E	E	D	D			
125	241									D	D	D	D	P	E	D	D	E	E	E	D	D	E	E	E				
125	242													E	D	D	D	E	E	M	D	D	E	E	D	D	E	E	D
125	243											E	D	D	D	D	P	E	D	D	D	P	E	D	D	P	E		
125	244										D	D	E	E	D	D	D	E	E	D	D	D	E	E	E	D			
125	245										D	D	E	M	D	D	D	E	E	D	D	D	E	D	D	D			
125	246										D	D	E	E	D	D	D	E	E	D	D	D	E	E	D	D			
125	247													E	D	D	P	E	E	D	D	E	E	E	D	D	E	E	D
125	248											E	E	E	D	D	D	E	E	D	D	D	E	E	D	D	D		
125	249												P	E	D	D	D	E	E	D	D	D	P	E					
125	250													E	D	D	D	E	E	D	D	D	P	E	D	D	D	E	E
250	251									D	D	D	E	E	D	D	D	E	E	D	D	D	D	D	D				
250	252							D	D	D	I	P	E	D	D	D	P	E	D	D	D	P	E						
250	253												D	E	D	D	D	E	E	E	D	D	D	E	E	D	D	D	
250	254			P	E	E	D	D	D	D	D	D	D	D	D	I	P	E											
250	255										D	P	E	E	D	D	D	E											
250	256										D	P	E	E	D	D	D	E	E	M	D	D	D	D	P	E			
250	257		D	D	D	D	D	I	E	D	D	D	M	D	D	D	D	D											
250	258		D	D	D	P	E	D	D	D	D	D	D	D	D	D	D	I											
250	259			D	E	E	E	D	D	D	D	D	D	D	D	D	D	D	E										
250	260											E	E	D	D	D	D	E	D	D	D	D	D	D	I	D	D		
500	261		D	D	D	D	D	D	D	D	D	P	E	D	M	D													
500	262		D	D	D	D	E	E	D	D	D																		
500	263		D	D	D	D	D	D	D	D																			
500	264		E	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D											
500	265		D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D											
500	266		D	D	D	D	D	D	D	D	D																		
500	267		M	D	D																								
500	268		D	D	D	D	D	D	D	D	D	D	D																
500	269		D	D	D	D	D	D	D	D																			
500	270		D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D											

Figure A-1. Vaginal Cytology Plots for Female Rats in the 28-day Gavage Study of Perfluorobutane Sulfonic Acid

I = insufficient number of cells to determine stage; D = diestrus; P = proestrus; E = estrus; M = metestrus. One-half the dose was administered twice daily.

Dose (mg/kg /day)	Animal ID																													
0	1061														E	D	D	E	E	E	D	D	D	E	E	D	D	E	E	E
0	1062								E	D	D	D	D	D	D	D	D	E	E	D	P	E	E	D						
0	1063									D	D	E	E	M	D	D	P	E	M	D	D	P	E	E	D					
0	1064											D	E	E	D	D	P	E	E	D	D	P	E	E	D	D	P			
0	1065											P	E	E	D	D	D	E	E	D	D	D	D	E	D	D	P			
0	1066									D	D	D	E	E	D	D	P	E	E	D	D	E	E	E	D					
0	1067									D	D	D	E	E	D	D	D	E	E	D	D	D	D	E	D					
0	1068											P	E	E	D	D	P	E	D	D	P	E	E	D	D	D	E			
0	1069												E	E	D	D	D	E	E	M	D	P	E	E	D	D	D	E		
0	1070													E	E	D	D	E	E	E	D	D	D	E	E	D	D	D	E	
12.5	1091													E	D	D	P	E	D	D	P	E	D	D	P	E	D	D	E	
12.5	1092												E	D	D	D	D	P	E	D	D	D	D	E	E	D	D	D		
12.5	1093											D	D	P	E	D	D	P	E	E	D	D	E	E	E	D	D			
12.5	1094											M	D	P	E	D	D	P	E	D	D	P	E	D	D	D	E			
12.5	1095												D	P	E	D	D	P	E	E	M	D	D	E	E	D	D	E		
12.5	1096							D	D	D	D	D	D	E	E	D	D	P	E	E	D	D	E							
12.5	1097										D	D	D	P	E	D	D	D	E	E	D	D	D	P	E	D				
12.5	1098												P	E	E	D	D	P	E	E	D	D	E	E	M	D	E	E		
12.5	1099								E	D	D	D	D	D	D	D	D	D	E	E	D	P	E	E						
12.5	1100										M	D	P	E	E	D	D	D	E	E	D	D	P	E	E	D				
25	1101										D	D	E	E	E	D	D	P	E	D	E	E	E	D	D	E				
25	1102		E	E	D	D	D	D	D	D	D	D	D	D	D	D	D	D												
25	1103											D	D	D	E	E	E	D	E	E	E	D	D	D	P	E	D			
25	1104											D	E	E	E	D	D	P	E	E	D	D	P	E	D	D	D			
25	1105										D	D	E	E	E	D	D	D	E	D	D	D	E	E	D	D				
25	1106														E	D	D	P	E	E	D	D	D	E	E	D	D	D	E	E
25	1107													E	E	D	D	P	E	E	D	D	E	E	E	D	D	P	E	
25	1108					M	D	D	D	D	D	D	D	D	E	D	D	P	E	E	D									
25	1109										D	D	E	E	E	D	D	P	E	E	D	D	D	E	E	D				
25	1110								D	E	D	D	D	D	D	D	D	D	E	E	D	D	P	E						
50	1111													E	D	D	D	E	E	D	D	P	E	E	D	D	D	E	E	
50	1112					M	D	D	D	D	D	D	D	P	E	D	D	P	E	E	D									
50	1113															E	D	E	D	D	P	E	D	D	E	E	M	D	E	D	D
50	1114											D	P	E	E	D	D	P	E	E	D	D	D	E	E	D	D			
50	1115								D	D	D	D	D	D	E	D	D	P	E	M	D	D	D	E						
50	1116												D	D	E	E	D	D	E	E	E	D	D	P	E	D	D	D		
50	1117								M	D	P	M	D	D	E	D	D	D	E	M	D	E	E	D						
50	1118												D	E	E	D	D	P	E	M	D	D	P	M	D	D	D	D		
50	1119													E	E	D	D	P	E	E	D	D	D	E	E	D	D	D	E	
50	1120										D	D	P	E	E	D	D	D	E	E	D	D	D	D	E	D				

Figure A-2. Vaginal Cytology Plots for Female Rats in the 28-day Gavage Study of Perfluorohexane Sulfonate Potassium Salt

D = diestrus; P = proestrus; E = estrus; M = metestrus.

Dose (mg/kg /day)	Animal ID																											
0	361		D	D	D	D	D	D	D	D	D	D	E	D	D	P	E	D										
0	362												E	M	D	D	E	E	D	D	D	P	E	D	D	D	P	E
0	363								D	D	E	E	E	D	D	E	E	D	D	D	P	E	D	I				
0	364											E	M	D	P	E	E	E	M	D	P	E	E	D	D	P	E	
0	365											E	D	D	D	E	E	D	D	E	E	D	D	D	E	E	D	
0	366											E	E	M	D	E	E	E	D	D	P	E	M	D	P	E	E	
0	367									E	E	E	D	D	P	E	E	E	D	D	D	D	D	E	E			
0	368									D	D	E	D	D	P	E	E	D	D	D	E	E	D	D	I			
0	369							D	D	P	E	D	D	D	P	E	D	D	D	E	M	D	D					
0	370							D	D	D	E	E	D	D	P	E	E	D	D	P	D	D	P					
0.625	381					P	M	M	D	P	E	E	D	D	D	E	E	D	D	P	M							
0.625	382							D	D	D	E	E	M	D	P	E	D	D	D	D	D	E	D					
0.625	383							M	M	D	E	E	D	D	D	E	E	D	D	D	P	E	D					
0.625	384									D	E	E	D	D	D	E	E	M	D	D	D	P	M	D	D			
0.625	385											E	D	D	P	E	E	D	D	E	E	D	D	D	E	E	D	
0.625	386								D	E	E	D	D	D	D	E	D	E	D	D	D	P	D	D				
0.625	387							D	D	E	E	D	D	D	P	E	D	D	D	E	E	D	D					
0.625	388								D	E	E	D	D	D	P	E	E	D	D	D	E	E	D	D				
0.625	389										E	D	D	D	P	E	D	D	D	P	E	D	D	D	P	E		
0.625	390							D	D	E	E	D	D	D	P	E	D	D	D	E	E	D	D					
1.25	391											E	M	D	P	E	E	E	M	D	P	E	E	D	D	P	E	
1.25	392									D	E	M	D	D	P	E	D	D	D	D	E	E	M	D	D			
1.25	393									E	E	D	D	D	P	E	D	D	D	E	E	M	D	P	E			
1.25	394							D	D	P	E	E	D	D	P	E	D	D	P	E	E	D	D					
1.25	395									D	P	E	D	D	D	E	E	D	D	D	E	E	D	D	D			
1.25	396						D	D	D	E	E	D	D	D	P	E	D	D	D	E	E	D						
1.25	397										E	E	D	D	D	E	E	D	D	D	P	E	D	D	P	E		
1.25	398							D	D	E	D	D	D	D	D	E	E	D	D	D	E	E	D					
1.25	399									D	E	E	D	D	P	E	E	D	D	D	E	E	D	D	D			
1.25	400											P	E	D	D	E	E	D	D	D	E	E	D	D	P	E	E	
2.5	401						M	D	D	D	E	E	D	D	D	E	E	D	D	D	P	E						
2.5	402								D	E	E	D	D	D	D	E	D	D	D	E	E	D	D	E				
2.5	403												E	D	D	E	E	D	D	P	E	E	D	D	D	P	E	D
2.5	404									D	E	E	D	D	D	E	E	D	D	D	E	E	D	D	E			
2.5	405							D	D	D	P	E	D	D	D	E	E	D	D	D	P	E	D					
2.5	406					D	D	D	P	E	E	D	D	D	P	E	E	D	D	E	E							
2.5	407											E	M	D	P	E	E	D	D	D	P	E	D	D	D	E	E	
2.5	408									P	E	D	D	D	P	E	D	D	D	P	E	D	D	D	D			
2.5	409								D	E	M	D	D	D	D	E	E	D	D	E	E	E	D	D				
2.5	410							D	E	E	D	D	D	D	P	E	D	D	D	E	E	D	D					

Figure A-3. Vaginal Cytology Plots for Female Rats in the 28-day Gavage Study of Perfluorooctane Sulfonic Acid

I = insufficient number of cells to determine stage; D = diestrus; P = proestrus; E = estrus; M = metestrus.

Dose (mg/kg /day)	Animal ID																													
0	491												D	E	E	E	E	D	D	E	E	D	D	D	E	E	E	D	D			
0	492		D	D	D	D	D	D	D	D	P	M	D	D	P	E	D	D													
0	493										D	D	E	E	E	D	D	D	E	E	D	D	D	E	E	D					
0	494									D	D	D	E	E	E	D	D	P	E	D	D	P	E	E	D						
0	495														E	D	D	D	E	E	E	E	D	D	D	E	E	D	D	D	
0	496												D	D	E	M	D	D	E	E	D	D	D	P	E	E	D	D			
0	497													P	E	D	D	D	E	E	M	D	D	D	E	E	D	D	D		
0	498													E	E	D	D	P	E	E	D	D	D	E	E	D	D	D	E		
0	499														E	D	D	D	E	E	D	D	D	E	D	D	E	E	D	D	
0	500												D	D	E	E	E	D	E	E	E	D	D	E	E	E	D	D			
6.25	501												D	E	E	D	D	D	E	E	D	D	D	E	E	D	D	D			
6.25	502										D	D	D	E	E	D	D	D	E	E	D	D	D	E	E	D					
6.25	503										D	D	D	E	E	D	D	D	E	E	D	D	D	P	E	D					
6.25	504															E	M	D	E	D	D	D	D	D	E	D	D	D	D	D	E
6.25	505									D	D	D	E	E	D	D	D	D	E	M	D	D	E	E	D						
6.25	506													E	E	D	D	D	E	E	D	D	D	E	E	E	D	P	E		
6.25	507												E	E	D	D	D	D	E	E	D	D	D	E	D	D	D	D			
6.25	508													D	E	M	D	D	E	D	D	D	E	E	D	D	D	D	P		
6.25	509										D	D	D	E	E	D	D	D	E	E	D	D	D	P	E	D					
6.25	510										D	D	D	P	E	D	D	D	E	E	D	D	D	P	E	D					
12.5	511												D	E	E	E	D	D	E	D	D	D	D	D	D	D	D	D			
12.5	512										D	D	D	P	E	M	D	D	E	E	D	D	D	D	E	M					
12.5	513										D	D	E	E	D	D	D	P	E	D	D	D	P	E	D	D					
12.5	514															E	D	D	E	E	E	D	D	D	P	E	D	D	D	P	E
12.5	515													P	E	D	D	P	E	E	D	D	D	P	E	D	D	P	E		
12.5	516												E	E	M	D	D	P	E	D	D	D	P	E	M	D	D	P			
12.5	517		D	D	D	D	D	D	D	D	D	D	D	D	E	D	D	P													
12.5	518														E	D	D	D	E	E	D	D	P	E	E	D	D	D	D	P	
12.5	519									D	P	E	M	D	D	D	D	I	E	D	D	D	P	E	D						
12.5	520														D	E	E	D	E	P	E	D	D	D	P	E	D	D	D	P	
25	521											D	E	D	D	D	D	P	E	D	D	D	D	P	E	D	D				
25	522											D	E	E	D	D	D	P	E	D	D	D	D	D	D	D	D				
25	523													E	E	D	D	D	E	E	D	D	D	P	E	D	D	D	P		
25	524													E	E	M	D	D	E	E	D	D	D	E	E	D	D	D	E		
25	525													E	D	D	D	D	E	E	D	D	D	E	E	M	D	D	D		
25	526													E	E	E	D	D	E	E	D	D	D	D	E	E	D	D	D		
25	527												E	E	D	D	D	P	E	D	D	D	P	E	M	D	D	D			
25	528											D	D	E	E	D	D	D	E	E	M	D	D	E	E	D	D				
25	529			D	D	D	D	D	P	D	D	P	E	D	D	D	D	P	E												
25	530												D	E	M	D	D	D	E	D	D	D	E	E	D	D	D	D			

Figure A-4. Vaginal Cytology Plots for Female Rats in the 28-day Gavage Study of Wyeth-14,643

I = insufficient number of cells to determine stage; D = diestrus; P = proestrus; E = estrus; M = metestrus.

Appendix B. Genetic Toxicology

Tables

Table B-1. Mutagenicity of Perfluorobutane Sulfonic Acid in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9	With 10% Rat S9
TA100						
	0	68 ± 3		57 ± 2	53 ± 2	
	50	51 ± 2				
	100	55 ± 1				
	200	53 ± 2				
	350	52 ± 12				
	500	59 ± 5		51 ± 2	63 ± 6	
	1,000			51 ± 1	53 ± 4	
	2,000			40 ± 1	50 ± 2	
	3,500			40 ± 2	47 ± 3	
	5,000			54 ± 3	70 ± 9	
Trial Summary		Negative		Negative	Negative	
Positive Control[b]		377 ± 22		648 ± 21	985 ± 39	
TA98						
	0	17 ± 2	12 ± 1	21 ± 4	29 ± 2	13 ± 2
	50	17 ± 2	46 ± 4			
	100	14 ± 2	37 ± 2			
	200	19 ± 8	27 ± 2			
	350	13 ± 1	41 ± 4			
	500	11 ± 2	29 ± 1	23 ± 1	22 ± 1	41 ± 4
	1,000			20 ± 3	14 ± 4	41 ± 1
	2,000			24 ± 1	16 ± 6	40 ± 6
	3,500			41 ± 2	21 ± 2	43 ± 3
	5,000			35 ± 9	41 ± 2	45 ± 8
Trial Summary		Negative	Positive	Equivocal	Negative	Positive
Positive Control		427 ± 4	170 ± 6	473 ± 20	826 ± 50	500 ± 28
***Escherichia coli WP2 uvrA*/pKM101**						
	0	229 ± 13	193 ± 7	229 ± 13	244 ± 9	
	50	212 ± 6	201 ± 11			
	100	228 ± 11	207 ± 7	289 ± 6	285 ± 15	
	200	211 ± 16	186 ± 6	311 ± 15	300 ± 14	
	350	165 ± 22	220 ± 11			
	500	217 ± 7	226 ± 9	262 ± 25	285 ± 3	
	750			268 ± 8	263 ± 4	
	1,000			273 ± 20		
Trial Summary		Negative	Negative	Negative	Negative	
Positive Control		663 ± 46	768 ± 5	713 ± 18	752 ± 7	

[a]Study was performed at ILS, Inc. Data are presented as revertants/plate (mean ± standard error) from three plates; 0 µg/plate was the solvent control.
[b]The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-o-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

Table B-2. Mutagenicity of Perfluorooctane Sulfonic Acid Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9
TA100					
	0	44 ± 7	68 ± 3	57 ± 2	49 ± 3
	100	56 ± 1	50 ± 5		
	250	43 ± 7	51 ± 5		
	500	32 ± 6	51 ± 8	54 ± 2	45 ± 1
	750	32 ± 2	51 ± 3		
	1,000	27 ± 6	37 ± 2	51 ± 1	52 ± 1
	2,000			42 ± 2	40 ± 1
	3,500			36 ± 0	36 ± 5
	5,000			21 ± 4	33 ± 5
Trial Summary		Negative	Negative	Negative	Negative
Positive Control[b]		385 ± 4	377 ± 22	648 ± 21	985 ± 39
TA98					
	0	14 ± 1	17 ± 2	21 ± 4	29 ± 2
	100	22 ± 1	18 ± 0		
	250	30 ± 3	15 ± 1		
	500	31 ± 2	15 ± 3	31 ± 4	15 ± 3
	750	22 ± 3	17 ± 3		
	1,000	28 ± 2	18 ± 3	24 ± 2	13 ± 3
	2,000			25 ± 2	17 ± 4
	3,500			21 ± 2	19 ± 3
	5,000			15 ± 1	11 ± 1
Trial Summary		Equivocal	Negative	Negative	Negative
Positive Control		170 ± 6	427 ± 4	473 ± 20	826 ± 50
***Escherichia coli WP2 uvrA*/pKM101**					
	0	229 ± 13	178 ± 17	231 ± 13	242 ± 9
	100	207 ± 10	158 ± 22		
	250	202 ± 17	162 ± 5		
	500	172 ± 4	151 ± 6		
	750	196 ± 7	178 ± 5		
	1,000	209 ± 21	171 ± 13	255 ± 14	268 ± 12
	2,500			259 ± 2	257 ± 10
	5,000			234 ± 17	218 ± 40
	7,500			233 ± 17	221 ± 23
	10,000			227 ± 16	236 ± 11
Trial Summary		Negative	Negative	Negative	Negative
Positive Control		663 ± 46	768 ± 5	713 ± 18	752 ± 7

[a]Study was performed at ILS, Inc. Data are presented as revertants/plate (mean ± standard error) from three plates; 0 µg/plate was the solvent control.
[b]The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-o-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

Table B-3. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Rats Following Administration of Perfluorobutane Sulfonic Acid by Gavage for 28 Days[a]

	Dose[b] (mg/kg /day)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[c]	P Value[d]	Micronucleated NCEs/1,000 NCEs[c]	P Value[d]	PCEs (%)[c]	P Value[d]
Male								
Vehicle Control[e]		5	0.59 ± 0.11		0.33 ± 0.11		1.01 ± 0.06	
PFBS	62.6	5	0.50 ± 0.11	0.617	0.12 ± 0.01	1.000	0.93 ± 0.06	1.000
	125	5	0.62 ± 0.05	0.574	0.13 ± 0.03	1.000	0.91 ± 0.06	1.000
	250	5	0.62 ± 0.12	0.608	0.13 ± 0.02	1.000	0.83 ± 0.08	0.375
	500	5	0.57 ± 0.12	0.626	0.12 ± 0.02	1.000	0.53 ± 0.10	0.004
			p = 0.469[f]		p = 0.984		p = 0.000	
Female								
Vehicle Control		5	0.62 ± 0.12		0.06 ± 0.11		1.23 ± 0.16	
PFBS	62.6	5	0.51 ± 0.09	0.630	0.09 ± 0.02	0.159	0.78 ± 0.10	0.046
	125	5	0.63 ± 0.18	0.558	0.08 ± 0.02	0.192	0.80 ± 0.04	0.053
	250	5	0.74 ± 0.07	0.349	0.09 ± 0.01	0.206	0.70 ± 0.13	0.009
	500	5	0.70 ± 0.08	0.362	0.06 ± 0.01	0.213	0.53 ± 0.10	0.001
			p = 0.154		p = 0.647		p = 0.001	

PFBS = perfluorobutane sulfonic acid; PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte.
[a]Study was performed at ILS, Inc. The detailed protocol is presented by Witt et al.[101].
[b]One-half the dose was administered twice daily. No data were available for 1,000 mg/kg/day rats due to high mortality.
[c]Mean ± standard error.
[d]Pairwise comparison with the vehicle control group; dose group values for PCEs and NCEs are significant when the one-sided $p \leq 0.025$ by Williams' test. P values for % PCEs are compared to a two-sided $p \leq 0.05$.
[e]The vehicle control was 2% Tween® 80 in deionized water.
[f]Dose-related trend; significant when compared to one-sided $p \leq 0.025$ by linear regression or Jonckheere's test.

Table B-4. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Rats Following Administration of Perfluorohexane Sulfonate Potassium Salt by Gavage for 28 Days[a]

	Dose (mg/kg/day)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs (%)[b]	P Value[c]
Male								
Vehicle Control[d]		5	0.53 ± 0.06		0.05 ± 0.01		0.98 ± 0.07	
PFHxSK	0.625	5	0.62 ± 0.09	0.521	0.06 ± 0.02	1.000	0.78 ± 0.08	0.038
	1.25	5	0.48 ± 0.10	0.603	0.03 ± 0.01	1.000	0.74 ± 0.03	0.045
	2.5	5	0.65 ± 0.07	0.638	0.04 ± 0.01	1.000	0.81 ± 0.05	0.047
	5	5	0.37 ± 0.11	0.657	0.03 ± 0.00	1.000	0.69 ± 0.06	0.003
	10	5	0.47 ± 0.06	0.670	0.04 ± 0.01	1.000	0.67 ± 0.05	0.002
			p = 0.887[e]		p = 0.974		p = 0.007	
Female								
Vehicle Control		5	0.75 ± 0.08		0.12 ± 0.03		0.91 ± 0.12	
PFHxSK	3.12	5	0.67 ± 0.09	0.764	0.08 ± 0.02	0.828	1.27 ± 0.14	0.352
	6.25	5	0.54 ± 0.07	0.843	0.09 ± 0.01	0.897	0.94 ± 0.12	0.423
	12.5	5	0.76 ± 0.09	0.872	0.09 ± 0.02	0.918	1.03 ± 0.11	0.453
	25	5	0.61 ± 0.11	0.884	0.08 ± 0.01	0.928	0.98 ± 0.06	0.466
	50	5	0.59 ± 0.12	0.894	0.10 ± 0.03	0.836	1.01 ± 0.11	0.474
			p = 0.821		p = 0.445		p = 0.975	

PFHxSK = perfluorohexane sulfonate potassium salt; PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte.
[a]Study was performed at ILS, Inc. The detailed protocol is presented by Witt et al.[101].
[b]Mean ± standard error.
[c]Pairwise comparison with the vehicle control group; dose group values for PCEs and NCEs are significant when the one-sided $p \leq 0.025$ by Williams' test. P values for % PCEs are compared to a two-sided $p \leq 0.05$.
[d]The vehicle control was 2% Tween® 80 in deionized water.
[e]Dose-related trend; significant when compared to one-sided $p \leq 0.025$ by linear regression or Jonckheere's test.

Table B-5. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Rats Following Administration of Perfluorooctane Sulfonic Acid by Gavage for 28 Days[a]

	Dose (mg/kg /day)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs (%)[b]	P Value[c]
Male								
Vehicle Control[d]		5	0.50 ± 0.12		0.12 ± 0.03		0.83 ± 0.04	
PFOS	0.312	5	0.89 ± 0.14	0.077	0.14 ± 0.01	0.431	0.75 ± 0.06	0.482
	0.625	5	0.76 ± 0.08	0.092	0.16 ± 0.03	0.285	0.76 ± 0.08	0.568
	1.25	5	0.67 ± 0.05	0.098	0.08 ± 0.01	1.000	0.68 ± 0.05	0.227
	2.5	5	0.72 ± 0.05	0.099	0.07 ± 0.01	1.000	0.54 ± 0.07	0.003
	5	5	0.70 ± 0.20	0.101	0.10 ± 0.01	1.000	0.41 ± 0.04	0.000
			p = 0.466[e]		p = 0.961		p = 0.000	
Female								
Vehicle Control		5	0.43 ± 0.07		0.05 ± 0.00		0.93 ± 0.11	
PFOS	0.312	5	0.52 ± 0.09	0.248	0.06 ± 0.03	1.000	0.83 ± 0.12	0.961
	0.625	5	0.59 ± 0.10	0.200	0.03 ± 0.01	1.000	1.05 ± 0.10	0.997
	1.25	5	0.59 ± 0.08	0.215	0.03 ± 0.00	1.000	0.86 ± 0.09	0.763
	2.5	5	0.49 ± 0.07	0.221	0.02 ± 0.00	1.000	0.63 ± 0.05	0.024
	5	5	0.77 ± 0.13	0.009	0.03 ± 0.00	1.000	0.49 ± 0.07	0.000
			p = 0.014		p = 0.987		p = 0.000	

PFOS = perfluorooctane sulfonic acid; PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte.
[a]Study was performed at ILS, Inc. The detailed protocol is presented by Witt et al.[101].
[b]Mean ± standard error.
[c]Pairwise comparison with the vehicle control group; dose group values for PCEs and NCEs are significant when the one-sided $p \leq 0.025$ by Williams' test. P values for % PCEs are compared to a two-sided $p \leq 0.05$.
[d]The vehicle control was 2% Tween® 80 in deionized water.
[e]Dose-related trend; significant when compared to one-sided $p \leq 0.025$ by linear regression or Jonckheere's test.

Table B-6. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Rats Following Exposure to Wyeth-14,643 by Gavage for 28 Days[a]

	Dose (mg/kg /day)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs[b] (%)	P Value[c]
Male								
Vehicle Control[d]		5	0.51 ± 0.09		0.08 ± 0.01		0.97 ± 0.05	
Wyeth-14,643	6.25	5	0.80 ± 0.06	0.055	0.16 ± 0.04	0.131	0.76 ± 0.02	1.000
	12.5	5	0.86 ± 0.20	0.050	0.29 ± 0.13	0.032	1.18 ± 0.15	0.236
	25	5	0.79 ± 0.08	0.053	0.12 ± 0.03	0.465	1.08 ± 0.07	0.249
			p = 0.096[e]		p = 0.126		p = 0.110	
Female								
Vehicle Control		5	0.47 ± 0.09		0.04 ± 0.00		1.10 ± 0.09	
Wyeth-14,643	6.25	5	0.73 ± 0.13	0.105	0.06 ± 0.01	0.056	0.95 ± 0.09	1.000
	12.5	5	0.58 ± 0.07	0.127	0.04 ± 0.01	0.889	1.29 ± 0.07	0.187
	25	5	0.62 ± 0.06	0.135	0.04 ± 0.01	0.946	1.40 ± 0.11	0.052
			p = 0.292		p = 0.446		p = 0.013	

PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte.
[a]Study was performed at ILS, Inc. The detailed protocol is presented by Witt et al.[101].
[b]Mean ± standard error.
[c]Pairwise comparison with the vehicle control group; dose group values for PCEs and NCEs are significant when the one-sided $p \leq 0.025$ by Williams' test. P values for % PCEs are compared to a two-sided $p \leq 0.05$.
[d]The vehicle control was 2% Tween® 80 in deionized water.
[e]Dose-related trend; significant when compared to one-sided $p \leq 0.025$ by linear regression or Jonckheere's test.

Appendix C. Chemical Characterization and Dose Formulation Studies

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C.1. Procurement and Characterization

C.1.1. Perfluoroalkyl Sulfonates and Wyeth-14,643

Perfluorobutane sulfonic acid (PFBS) was obtained from Sigma Aldrich (St. Louis, MO) in one lot (15414TE), perfluorohexane sulfonate potassium salt (PFHxSK) was obtained from Interchim (Montlucon Cedex, France) in one lot (230002), perflourooctane sulfonic acid (PFOS) was obtained from Matrix Scientific (Columbia, SC) in one lot (T20G), and Wyeth-14,643 (WY) was obtained from ChemSyn Laboratories (Lenexa, KS) in one lot (91-314-72-07/91-314-100-33A); all lots were used in the 28-day gavage studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory and the study laboratory at Battelle (Columbus, OH). Reports on analyses performed in support of the perfluorinated sulfonate studies are on file at the National Institute of Environmental Health Sciences.

Lot 15414TE (PFBS), a colorless liquid; lot 230002 (PFHxSK), a white solid; lot T20G (PFOS), off-white particles; and lot 91-314-72-07/91-314-100-33A (WY), a white powder, were identified using infrared (IR) spectroscopy, mass spectrometry (MS) (perfluoroalkyl sulfonates only), and proton (WY only), carbon-13, and fluorine-19 (perfluoroalkyl sulfonates only) nuclear magnetic resonance (NMR) spectroscopy. The Advanced Chemistry Development (ACD) spectral prediction program (Version 10.02) was used to predict carbon-13 NMR spectra for each test chemical and proton NMR spectra for WY. Annotated fluorine-fluorine correlated spectroscopy (COSY) two-dimensional data were used to identify the fluorine-fluorine coupling within the perfluoroalkyl sulfonates by the observation of cross-peaks in each fluorine-19 NMR spectrum. For the WY molecule, COSY was used to identify proton-proton coupling, a distortionless enhancement by polarization transfer carbon-13 spectrum series was constructed to determine the types of carbon (methine, methylene, and methyl), and a proton-carbon-13 heteronuclear multiple quantum coherence two-dimensional spectrum was acquired to identify directly bonded protons and carbons. Spectra were in good agreement with the proposed structure of each test chemical, with ACD computer-predicted spectra, and with expected COSY-identified proton-proton and fluorine-fluorine coupling. Observed chemical shifts were in good agreement with those expected and reported for each test article. Representative IR spectra of lots 15414TE, 230002, T20G, and 91-314-72-07/91-314-100-33A are presented in Figure C-1, Figure C-2, Figure C-3, and Figure C-4, respectively.

The purities of lots 15414TE, 230002, and T20G were determined by high-performance liquid chromatography (HPLC)/ion chromatography (IC) with suppressed conductivity (SC) detection, HPLC with MS detection, and proton-induced X-ray emission (PIXE) spectroscopy (measuring 72 elements from sodium to uranium). The purity of lot 91-314-72-07/91-314-100-33A was determined by HPLC with ultraviolet light (UV) detection. Differential scanning calorimetry (DSC) was used to determine the purities of lots T20G and 91-314-72-07/91-314-100-33A using a Perkin-Elmer (Shelton, CT) Diamond differential scanning calorimeter scanning at 1°C per minute over the ranges of 20°C to 130°C, and 135°C to 175°C, respectively. In addition, Karl Fischer titration for lots 15414TE and T20G was performed by Galbraith Laboratories (Knoxville, TN).

For lot 15414TE (PFBS), HPLC/IC/SC analysis using System A (Table C-1) indicated one major peak that was 97.7% of the total peak area and one impurity (2.3% of the total peak area). HPLC/MS analysis using System B indicated one major peak and three impurities with a

combined area of 1.3% of the total peak area. PIXE analyses for carbon, hydrogen, oxygen, fluorine, and sulfur were in agreement with the theoretical values for PFBS and indicated the absence of significant inorganic impurities. Karl Fischer titration indicated 0.46% water. The overall purity of lot 15414TE was determined to be greater than 97%.

For lot 230002 (PFHxSK), HPLC/IC/SC analysis using System C indicated one major peak that was 99.9% of the total peak area and one impurity (0.1% of the total peak area). HPLC/MS using a system similar to System B indicated one major peak (98.6% of the total peak area) and four reportable impurities with areas ≥0.1% of the total peak area. Three of the impurities were identified as PFBS (0.28% of the total peak area), perfluoropentane sulfonic acid (0.42% of the total peak area), and PFOS (0.19% of the total peak area); one impurity (0.49% of the total peak area) was not identified. PIXE analyses for potassium and sulfur were in agreement with the theoretical values for PFHxSK and indicated the absence of significant inorganic impurities. The overall purity of lot 230002 was determined to be greater than 98%.

For lot T20G (PFOS), HPLC/IC/SC analysis using System C indicated one major peak (96.7% of the total peak area) and one impurity that represented 3.3% of the total peak area. HPLC/MS using a system similar to System B indicated one major peak (68.3% of the total peak area) and nine other peaks with areas ≥0.1% of the total peak area. Three of the nine peaks were identified as PFOS isomers (cumulatively 27.7% of the total peak area). From the remaining peaks, two (cumulatively 2.5% of the total peak area) were identified as perfluoroheptane sulfonic acid isomers and one (0.4% of the total peak area) was identified as a perfluorohexane sulfonic acid isomer. PIXE analyses for carbon, hydrogen, oxygen, fluorine, and sulfur were in agreement with the theoretical values for PFOS and indicated the absence of significant inorganic impurities. DSC indicated an average purity of 98.8%. Karl Fischer titration indicated 4.63% water. The overall purity of lot T20G was determined to be greater than 96% on the basis of PFOS total isomers.

For lot 91-314-72-07/91-314-100-33A (WY), HPLC/UV analysis using System E indicated one major peak (99.4% of the total peak area) and two impurities with areas ≥0.1% of the total peak area. DSC indicated an average purity of 94.3%; this lower calculated purity compared to that shown by HPLC was considered an indication of thermal decomposition rather than impurity of the bulk chemical. The overall purity of lot 91-314-72-07/91-314-100-33A was determined to be greater than 99%.

Stability studies of lots 15414TE (PFBS) and T20G (PFOS) of the bulk chemicals were performed by the analytical chemistry laboratory using HPLC/IC/SC by Systems A and C, respectively. Stability was confirmed for bulk chemical samples stored in sealed amber glass bottles at temperatures up to 60°C for at least 14 days. To ensure stability, the bulk chemicals were stored at refrigerated temperature (lot 15414TE), room temperature (lots 230002 and 91-314-72-07/91-314-100-33A), or −20°C (lot T20G) in sealed amber glass containers under nitrogen (PFBS only). Reanalyses of the bulk chemicals were performed by the study laboratory on December 31, 2011 (lot 15414TE), January 26, 2012 (lot 230002), January 1, 2012 (lot T20G), and January 10, 2012 (lot 91-314-72-07/91-314-100-33A) (prior to the gavage studies) using HPLC/IC/SC or HPLC/UV by Systems A, C, C, and E, respectively, and no degradation of the bulk chemicals was detected.

C.1.2. Tween® 80

Tween® 80 (polysorbate 80) was obtained from Spectrum Laboratory Products, Inc. (Gardena, CA), and was used at a 2% concentration as the vehicle in the 28-day gavage studies. The vehicle was prepared by mixing the appropriate amount of Tween 80 with deionized water in a calibrated carboy and stirring with an overhead stirrer until all the Tween 80 was dissolved.

C.2. Preparation and Analysis of Dose Formulations

The dose formulations were prepared by mixing PFBS, PFHxSK, PFOS, or WY with 2% Tween 80 in deionized water to give the required concentrations (Table C-2). Each formulation pH was adjusted to between 6 and 8. The dose formulations were stored at room (PFBS and PFHxSK) or refrigerated (PFOS and WY) temperatures in amber glass bottles sealed with Teflon®-lined lids for no more than 42 days.

Homogeneity studies of the 12.5 and 100 mg/mL dose formulations of PFBS and a stability study of the 12.5 mg/mL dose formulation were performed by the analytical chemistry laboratory using HPLC/IC/SC System A (Table C-1). Homogeneity was confirmed and stability was confirmed for at least 42 days for dose formulations stored in amber glass bottles sealed with Teflon-lined lids at room temperature, and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of a 0.12 mg/mL formulation and the 10 mg/mL dose formulation of PFHxSK and a stability study of a 0.12 mg/mL formulation were performed by the analytical chemistry laboratory using HPLC/IC/SC System D. Homogeneity was confirmed and stability was confirmed for at least 42 days for formulations stored in amber glass bottles sealed with Teflon-lined lids at room temperature, and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of a 0.06 mg/mL formulation and the 1 mg/mL dose formulation of PFOS and a stability study of a 0.06 mg/mL formulation were performed by the analytical chemistry laboratory using HPLC/IC/SC System D. Homogeneity was confirmed, and stability was confirmed for at least 41 days for formulations stored in amber glass bottles sealed with Teflon-lined lids at refrigerated temperature and for at least 3 hours under simulated animal room conditions.

Homogeneity studies of the 1.26 and 5 mg/mL dose formulations of WY and a stability study of the 1.26 mg/mL dose formulation were performed by the analytical chemistry laboratory using HPLC/UV System E. Homogeneity was confirmed, and stability was confirmed for at least 42 days for formulations stored in amber glass bottles sealed with Teflon-lined lids at refrigerated temperature and for at least 3 hours under simulated animal room conditions.

Analyses of the dose formulations for the 28-day studies of PFBS, PFHxSK, PFOS, and WY were conducted once for each study by the study laboratory using HPLC/IC/SC or HPLC/UV similar to Systems A, D, D, and E, respectively. All five dose formulations for the PFBS study were within 10% of the target concentrations (Table C-3). Animal room samples of these dose formulations were also analyzed; all six were within 10% of the target concentrations. For the PFHxSK study, all 10 dose formulations and all 10 animal room samples were within 10% of the target concentrations. For the PFOS study, all five dose formulations and all five of the animal

room samples were within 10% of the target concentrations. For the WY study, all three dose formulations and all three of the animal room samples were within 10% of the target concentrations.

Table C-1. High-performance Liquid Chromatography Systems Used in the 28-day Gavage Studies of Perfluoroalkyl Sulfonates and Wyeth-14,643[a]

Detection System	Column	Solvent System
System A		
Suppressed Conductivity	IonPac™ NS1, 150 mm × 4 mm, 5 µm particle size (Dionex Corporation, Sunnyvale, CA)	A) 0.1 M NaOH in water, B) 70% isopropanol in water, and C) water (4%A:28%B:68%C), isocratic; flow rate 0.6 mL/minute
System B		
Mass Spectrometry	Luna® C18, 150 mm × 4.6 mm, 5 µm particle size (Phenomenex, Torrance, CA)	A) 2 mM ammonium acetate, and B) 0.1% formic acid in acetonitrile; linear gradient from 49%A:51%B for 68 minutes to 40%A:60%B in 2 minutes, held for 10 minutes, then to 49%A:51%B in 0.1 minute, held for 99.9 minutes; flow rate 1.0 mL/minute
System C		
Suppressed Conductivity	IonPac™ NS1, 150 mm × 4 mm, 5 µm particle size (Dionex Corporation)	A) 0.1 M NaOH in water, B) 70% isopropanol in water, and C) water; linear gradient from 1%A:30%B:69%C for 3 minutes to 1%A:43% B:56%C in 14 minutes, held for 3 minutes, then to 1%A:30%B:69%C in 1 minute, held for 14 minutes; flow rate 0.6 mL/minute
System D		
Suppressed Conductivity	IonPac™ NS1, 150 mm × 4 mm, 5 µm particle size (Dionex Corporation)	A) 0.1 M NaOH in water, B) 70% isopropanol in water, and C) water; linear gradient from 1%A:30%B:69%C for 3 minutes to 1%A:50%B:49%C in 14 minutes, held for 3 minutes, then to 1%A:30%B:69%C in 1 minute, held for 14 minutes; flow rate 0.6 mL/minute
System E		
Ultraviolet (254 nm) Light	Inertsil® ODS-2, 150 mm × 4.6 mm (Phenomenex)	7.5 mM heptane sulfonic acid (pH 3.4) in water: methanol (32:68), isocratic; flow rate 1 mL/minute

[a]The high-performance liquid chromatographs were manufactured by Hitachi High-Technologies Science America, Inc. (Chatsworth, CA) (Systems A, C, and D), Agilent Technologies, Inc. (Palo Alto, CA) (System B) or Agilent Technologies, Inc. (Santa Clara, CA) or Waters Corporation (Milford, MA) (System E). The mass spectrometer was manufactured by Micromass® Quattro LC (Manchester, UK) (System B).

Table C-2. Preparation and Storage of Dose Formulations in the 28-day Gavage Studies of Perfluoroalkyl Sulfonates and Wyeth-14,643

Perfluorobutane Sulfonic Acid	Perfluorohexane Sulfonate Potassium Salt	Perfluorooctane Sulfonic Acid	Wyeth-14,643
Preparation			
A glove bag was set up with a nitrogen atmosphere inside a fume hood. While in a nitrogen atmosphere, the appropriate amount of perfluorobutane sulfonic acid was weighed inside the glove bag and transferred into a calibrated mixing container containing a stir bar. Approximately 80% of the final total vehicle volume of 2% Tween® 80 in deionized water was added to the mixing container, while in a nitrogen atmosphere, and the formulation was stirred on a stir plate to wet the test article. The mixing container was capped, removed from the glove bag and sonicated for approximately 15 minutes. The formulation was then stirred an additional 15 minutes on a stir plate. If necessary, the pH was adjusted to between 6 and 8 with 10 N, and/or 1 N, and/or 0.1 N NaOH; the formulation was then diluted to final volume with vehicle and stirred for an additional 15 minutes.	The appropriate amount of perfluorohexane sulfonic acid potassium salt was weighed and quantitatively transferred into a calibrated mixing container containing a stir bar, and approximately 90% of the final total vehicle volume of 2% Tween 80 in deionized water was added to the mixing container, and the formulation was stirred on a stir plate for approximately 15 minutes. If necessary, the pH was adjusted to between 6 and 8 with 10 N, and/or 1 N and/or 0.1 N NaOH, or 0.1 N HCl; the formulation was then diluted to volume with vehicle and stirred for an additional 15 minutes.	The appropriate amount of perfluorooctane sulfonic acid was weighed and quantitatively transferred into a calibrated mixing container containing a stir bar, and approximately 90% of the final total vehicle volume of 2% Tween 80 in deionized water was added to the mixing container and the formulation was stirred on a stir plate for approximately 15 minutes. If necessary, the pH was adjusted to between 6 and 8 with 10 N, and/or 1 N and/or 0.1 N NaOH; the formulation was then diluted to final volume with vehicle and stirred for an additional 15 minutes.	The appropriate amount of Wyeth-14,643 was weighed and quantitatively transferred into a calibrated mixing container containing a stir bar, and approximately 90% of the final total vehicle volume of 2% Tween 80 in deionized water was added to the mixing container and the formulation was stirred on a stir plate and/or sonicated for approximately 15 minutes. If necessary, the pH was adjusted to between 6 and 8 with 10 N, and/or 1 N, and/or 0.1 N NaOH; the formulation was then diluted to final volume with vehicle and stirred and/or sonicated for an additional 15 minutes.
Chemical Lot Number			
15414TE	230002	T20G	91-314-72-07/91-314-100-33A
Maximum Storage Time			
38 days	39 days	42 days	39 days

Perfluorobutane Sulfonic Acid	Perfluorohexane Sulfonate Potassium Salt	Perfluorooctane Sulfonic Acid	Wyeth-14,643
Storage Conditions			
Stored in amber glass bottles sealed with Teflon®-lined lids at room temperature	Stored in amber glass bottles sealed with Teflon-lined lids at room temperature	Stored in amber glass bottles sealed with Teflon-lined lids at 5°C	Stored in amber glass bottles sealed with Teflon-lined lids at 5°C
Study Laboratory			
Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)

Table C-3. Results of Analyses of Dose Formulations Administered to Rats in the 28-day Gavage Studies of Perfluoroalkyl Sulfonates and Wyeth-14,643

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Perfluorobutane Sulfonic Acid				
January 10, 2012	January 12, 2012	6.26	6.23	0
		12.5	12.4	−1
		25	24.8	−1
		50	49.4	−1
		100	101	+1
	February 20, 2012[b]	6.26	6.45	+3
		6.26	6.52	+4
		12.5	13.1	+5
		25	25.7	+3
		50	51.4	+3
		100	106	+6
Perfluorohexane Sulfonate Potassium Salt				
January 30, 2012	January 31, 2012	0.125	0.121	−3
		0.25	0.227	−9
		0.5	0.466	−7
		0.624	0.603	−3
		1	0.990	−1
		1.25	1.24	−1
		2	2.04	+2
		2.5	2.54	+2
		5	5.11	+2
		10	10.1	+1
	March 8, 2012[b]			
		0.125	0.122	−2
		0.25	0.233	−7
		0.5	0.476	−5
		0.624	0.599	−4
		1	0.980	−2
		1.25	1.23	−2
		2	2.01	1
		2.5	2.50	0
		5	5.02	0
		10	9.88	−1

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Perfluorooctane Sulfonic Acid				
January 11, 2012	January 13, 2012	0.0624	0.0573	−8
		0.125	0.114	−9
		0.25	0.232	−7
		0.5	0.483	−3
		1	1.01	+1
	February 23, 2012[b]	0.0624	0.0595	−5
		0.125	0.116	−7
		0.25	0.239	−4
		0.5	0.507	+1
		1	1.06	+6
Wyeth-14,643				
January 16, 2012	January 18, 2012	1.25	1.24	−1
		2.5	2.48	−1
		5	4.95	−1
	March 1, 2012[b]	1.25	1.25	0
		2.5	2.49	0
		5	4.94	−1

[a]Results of triplicate analyses. Dosing volume for all studies = 5.0 mL/kg. One-half the perfluorobutane sulfonic acid formulations were administered twice per day; perfluorohexane sulfonate potassium salt, perfluorooctane sulfonic acid, and Wyeth-14,643 formulations were administered once per day. For the perfluorobutane sulfonic acid study, 6.26 mg/mL = 62.6 mg/kg/day, 12.5 mg/mL = 125 mg/kg/day, 25 mg/mL = 250 mg/kg/day, 50 mg/mL = 500 mg/kg/day, and 100 mg/mL = 1,000 mg/kg/day. For males in the perfluorohexane sulfonate potassium salt study, 0.125 mg/mL = 0.625 mg/kg/day, 0.25 mg/mL = 1.25 mg/kg/day, 0.5 mg/mL = 2.5 mg/kg/day, 1 mg/mL = 5 mg/kg/day, and 2 mg/mL = 10 mg/kg/day. For females in the perfluorohexane sulfonate potassium salt study, 0.624 mg/mL = 3.12 mg/kg/day, 1.25 mg/mL = 6.25 mg/kg/day, 2.5 mg/mL = 12.5 mg/kg/day, 5 mg/mL = 25 mg/kg/day, and 10 mg/mL = 50 mg/kg/day. For the perfluorooctane sulfonic acid study, 0.0624 mg/mL = 0.312 mg/kg/day, 0.125 mg/mL = 0.625 mg/kg/day, 0.25 mg/mL = 1.25 mg/kg/day, 0.5 mg/mL = 2.5 mg/kg/day, and 1 mg/mL = 5 mg/kg/day. For the Wyeth-14,643 study, 1.25 mg/mL = 6.25 mg/kg/day, 2.5 mg/mL = 12.5 mg/kg/day, and 5 mg/mL = 25 mg/kg/day.
[b]Animal room samples.

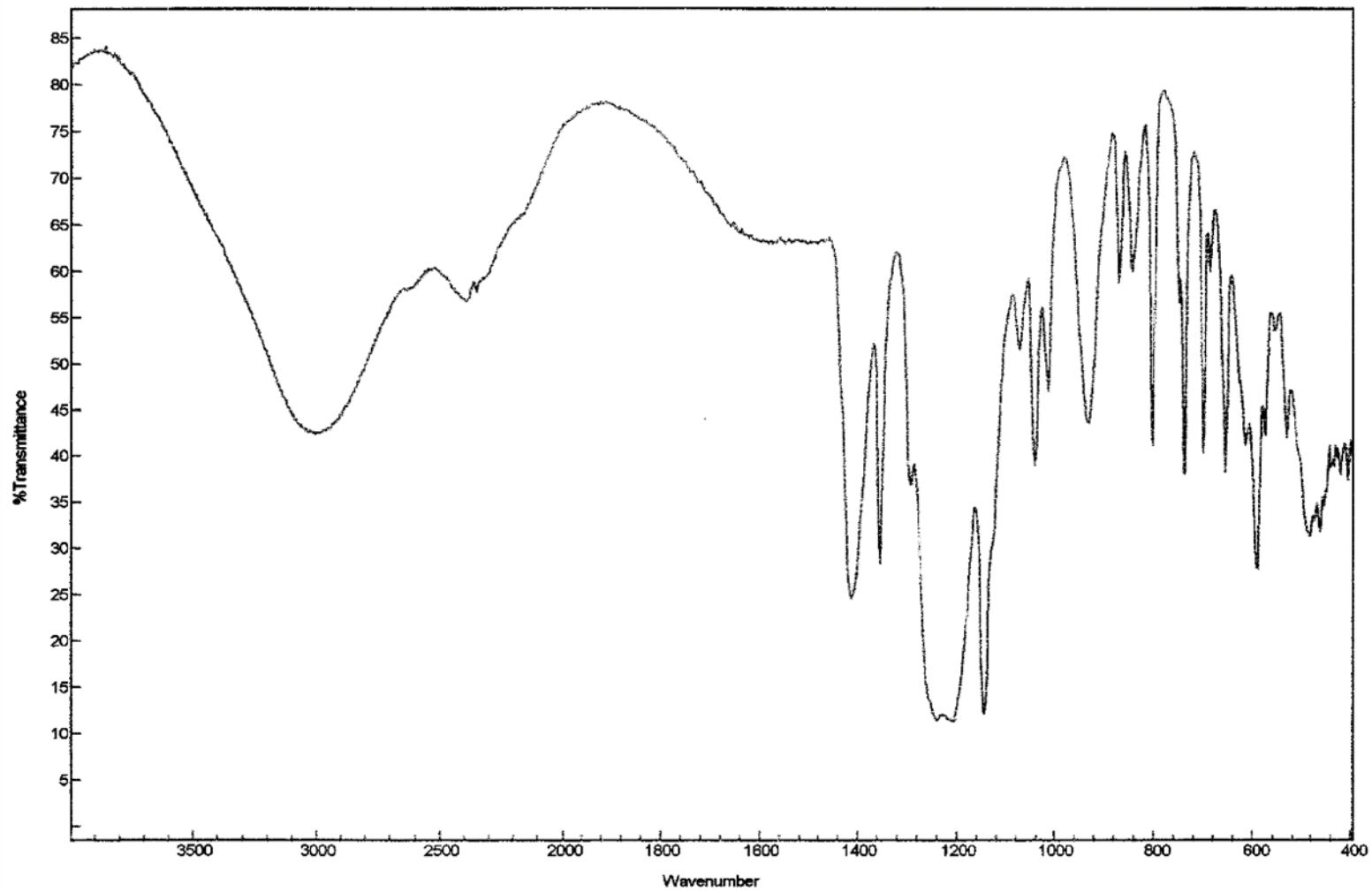

Figure C-1. Infrared Absorption Spectrum of Perfluorobutane Sulfonic Acid

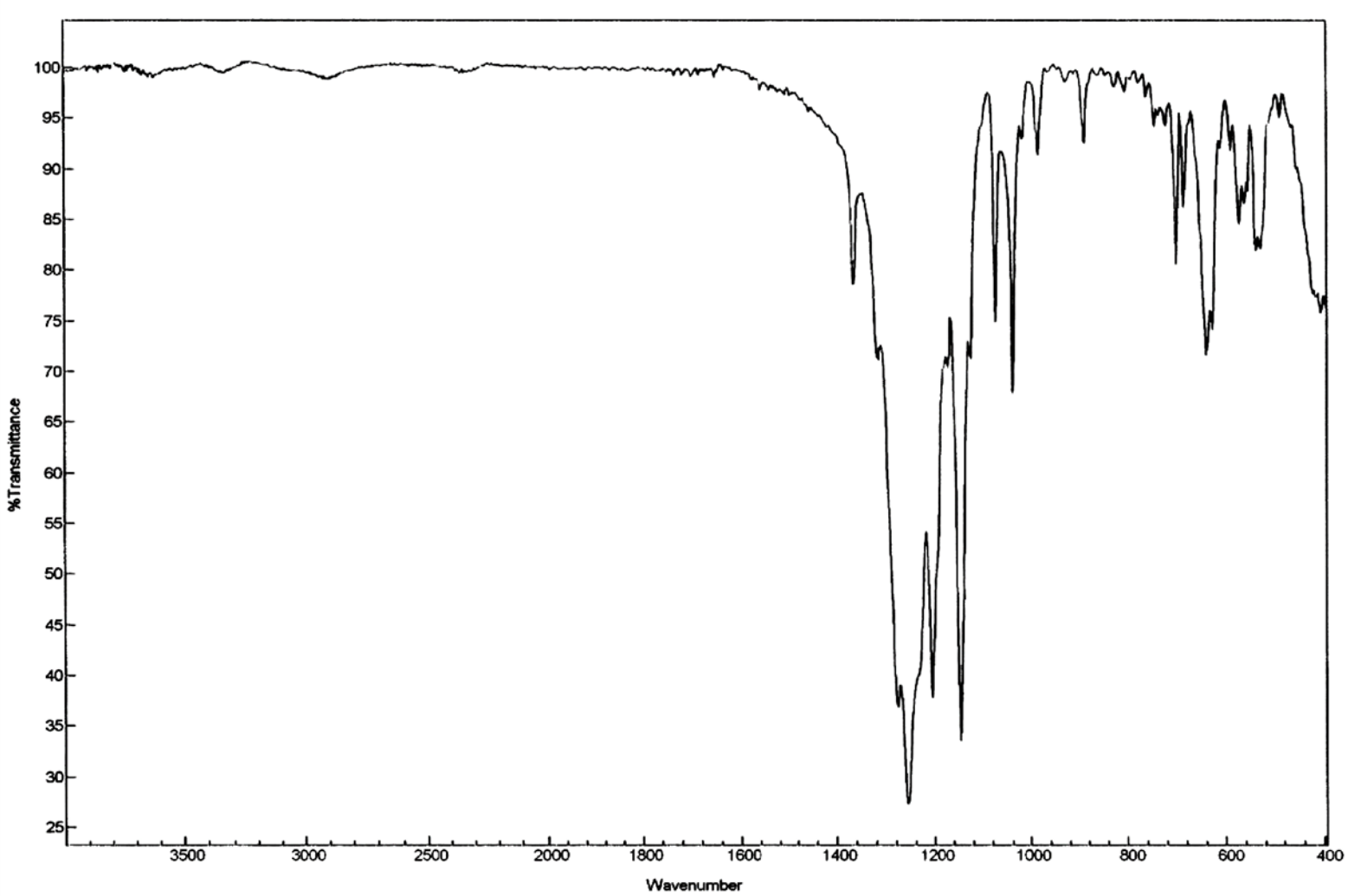

Figure C-2. Infrared Absorption Spectrum of Perfluorohexane Sulfonate Potassium Salt

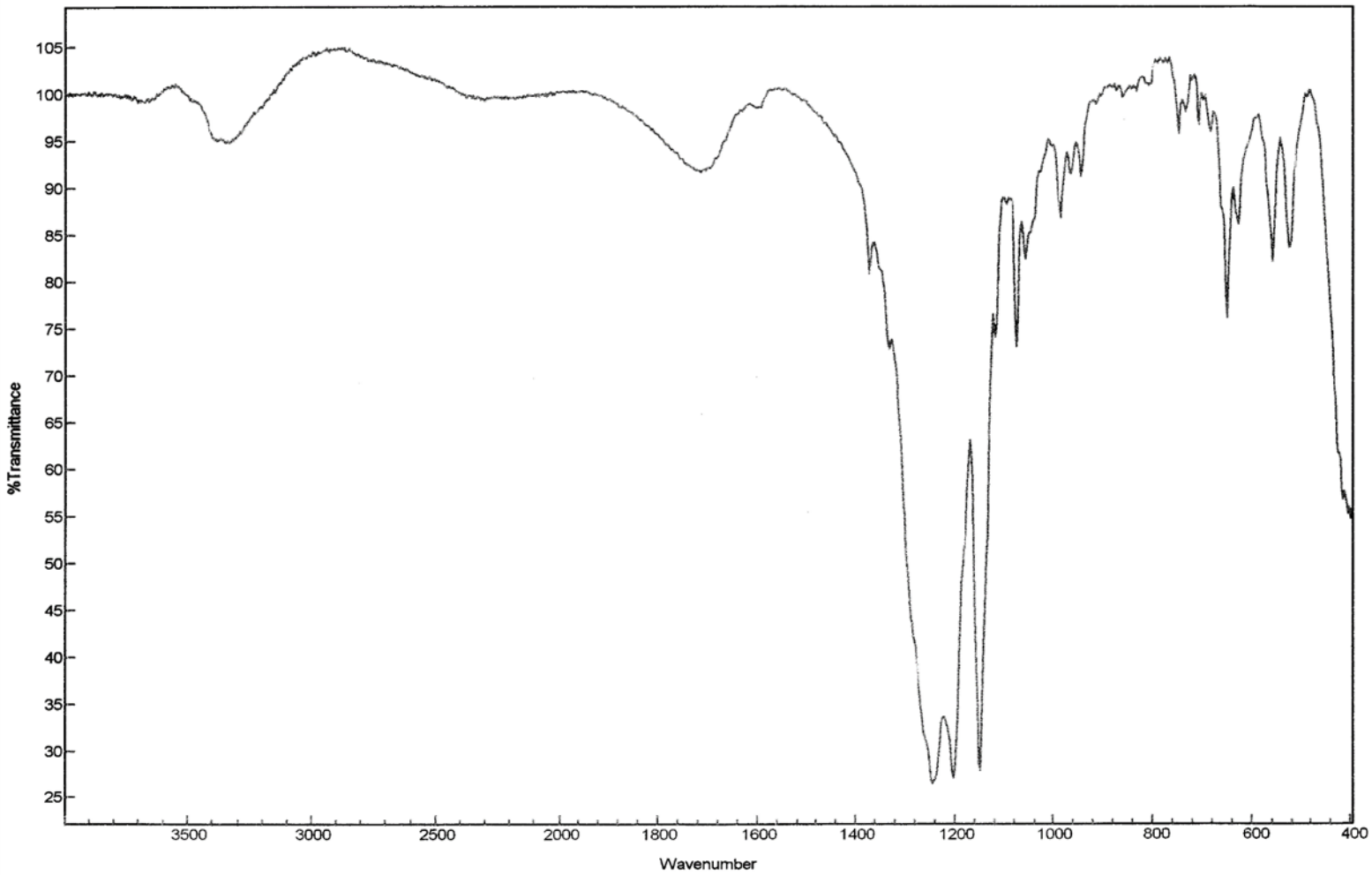

Figure C-3. Infrared Absorption Spectrum of Perfluorooctane Sulfonic Acid

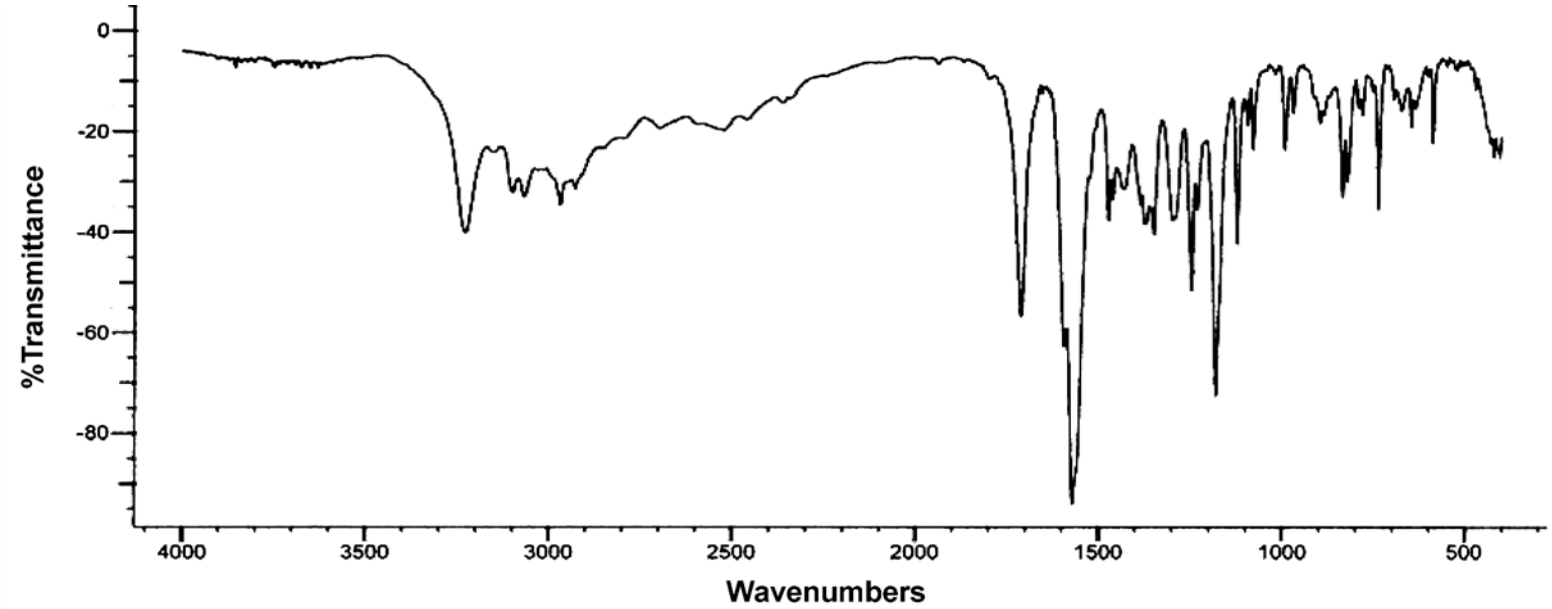

Figure C-4. Infrared Absorption Spectrum of Wyeth-14,643

Appendix D. Ingredients, Nutrient Composition, and Contaminant Levels in NTP-2000 Rat and Mouse Ration

Tables

Table D-1. Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground Hard Winter Wheat	22.26
Ground #2 Yellow Shelled Corn	22.18
Wheat Middlings	15.0
Oat Hulls	8.5
Alfalfa Meal (Dehydrated, 17% Protein)	7.5
Purified Cellulose	5.5
Soybean Meal (49% Protein)	5.0
Fish Meal (60% Protein)	4.0
Corn Oil (without Preservatives)	3.0
Soy Oil (without Preservatives)	3.0
Dried Brewer's Yeast	1.0
Calcium Carbonate (USP)	0.9
Vitamin Premix[a]	0.5
Mineral Premix[b]	0.5
Calcium Phosphate, Dibasic (USP)	0.4
Sodium Chloride	0.3
Choline Chloride (70% Choline)	0.26
Methionine	0.2

USP = United States Pharmacopeia.
[a]Wheat middlings as carrier.
[b]Calcium carbonate as carrier.

Table D-2. Vitamins and Minerals in NTP-2000 Rat and Mouse Ration[a]

	Amount	Source
Vitamins		
A	4,000 IU	Stabilized vitamin A palmitate or acetate
D	1,000 IU	D-activated animal sterol
K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl Acetate	100 IU	–
Niacin	23 mg	–
Folic Acid	1.1 mg	–
α-Pantothenic Acid	10 mg	α-Calcium pantothenate
Riboflavin	3.3 mg	–
Thiamine	4 mg	Thiamine mononitrate
B_{12}	52 µg	–
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	α-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a]Per kg of finished product.

Table D-3. Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	15.47 ± 1.05	14.4–16.5	3
Crude Fat (% by weight)	8.7 ± 0.10	8.6–8.8	3
Crude Fiber (% by weight)	9.73 ± 0.44	9.25–10.1	3
Ash (% by weight)	4.96 ± 0.14	4.80–5.04	3
Amino Acids (% of Total Diet)			
Arginine	0.802 ± 0.075	0.67–0.97	28
Cystine	0.220 ± 0.022	0.15–0.25	28
Glycine	0.703 ± 0.038	0.62–0.80	28
Histidine	0.342 ± 0.071	0.27–0.68	28
Isoleucine	0.549 ± 0.041	0.43–0.66	28
Leucine	1.097 ± 0.064	0.96–1.24	28
Lysine	0.700 ± 0.106	0.31–0.86	28
Methionine	0.410 ± 0.042	0.26–0.49	28
Phenylalanine	0.623 ± 0.047	0.47–0.72	28
Threonine	0.512 ± 0.042	0.43–0.61	28
Tryptophan	0.155 ± 0.027	0.11–0.20	28
Tyrosine	0.420 ± 0.066	0.28–0.54	28
Valine	0.666 ± 0.040	0.55–0.73	28
Essential Fatty Acids (% of Total Diet)			
Linoleic	3.88 ± 0.455	1.89–4.55	28
Linolenic	0.30 ± 0.065	0.007–0.368	28
Vitamins			
Vitamin A (IU/kg)	3,073 ± 49	2,520–3,450	3
Vitamin D (IU/kg)	1,000[a]	–	–
α-Tocopherol (ppm)	2,543 ± 13,044	27.0–69,100	28
Thiamine (ppm)[b]	7.53 ± 0.51	7.1–8.1	3
Riboflavin (ppm)	8.06 ± 2.83	4.20–17.50	28
Niacin (ppm)	78.6 ± 8.26	66.4–98.2	28
Pantothenic Acid (ppm)	26.6 ± 11.22	17.4–81.0	28
Pyridoxine (ppm)b	9.78 ± 2.08	6.44–14.3	28
Folic Acid (ppm)	1.58 ± 0.44	1.15–3.27	28
Biotin (ppm)	0.32 ± 0.09	0.20–0.704	28
Vitamin B_{12} (ppb)	50.6 ± 35.5	18.3–174.0	28
Choline (ppm)b	2,615 ± 635	1,160–3,790	28

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Minerals			
Calcium (%)	0.921 ± 0.021	0.906–0.945	3
Phosphorus (%)	0.564 ± 0.017	0.549–0.582	3
Potassium (%)	0.667 ± 0.030	0.626–0.733	28
Chloride (%)	0.393 ± 0.045	0.300–0.517	28
Sodium (%)	0.197 ± 0.026	0.160–0.283	28
Magnesium (%)	0.217 ± 0.055	0.185–0.490	28
Sulfur (%)	0.170 ± 0.029	0.116–0.209	14
Iron (ppm)	191.6 ± 36.8	135–311	28
Manganese (ppm)	50.1 ± 9.59	21.0–73.1	28
Zinc (ppm)	57.4 ± 26.0	43.3–184.0	28
Copper (ppm)	7.53 ± 2.53	3.21–16.3	28
Iodine (ppm)	0.531 ± 0.201	0.158–0.972	28
Chromium (ppm)	0.684 ± 0.258	0.330–1.380	27
Cobalt (ppm)	0.225 ± 0.154	0.086–0.864	26

[a]From formulation.
[b]As hydrochloride (thiamine and pyridoxine) or chloride (choline).

Table D-4. Contaminant Levels in NTP-2000 Rat and Mouse Ration[a]

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.1993 ± 0.047	0.16–0.25	3
Cadmium (ppm)	0.0507 ± 0.006	0.050–0.051	3
Lead (ppm)	0.072 ± 0.012	0.06–0.08	3
Mercury (ppm)	<0.02	–	3
Selenium (ppm)	0.122 ± 0.081	0.29–0.177	3
Aflatoxins (ppb)	<5.00	–	3
Nitrate Nitrogen (ppm)[c]	12.27 ± 3.4	10.0–16.2	3
Nitrite Nitrogen (ppm)[c]	<0.61	–	3
BHA (ppm)[d]	<1.0	–	3
BHT (ppm)[d]	<1.0	–	3
Aerobic Plate Count (CFU/g)	<10.0	–	3
Coliform (MPN/g)	3.0	–	3
Escherichia coli (MPN/g)	<10	–	3
Salmonella (MPN/g)	Negative	–	3
Total Nitrosoamines (ppb)[e]	10.7 ± 6.8	5.0–18.2	3
N-Nitrosodimethylamine (ppb)[e]	0.8 ± 0.7	0–1.3	3
N-Nitrosopyrrolidine (ppb)[e]	10 ± 6.2	5.0–17.0	3
Pesticides (ppm)			
α-BHC	<0.01	–	3
β-BHC	<0.02	–	3
γ-BHC	<0.01	–	3
δ-BHC	<0.01	–	3
Heptachlor	<0.01	–	3
Aldrin	<0.01	–	3
Heptachlor Epoxide	<0.01	–	3
DDE	<0.01	–	3
DDD	<0.01	–	3
DDT	<0.01	–	3
HCB	<0.01	–	3
Mirex	<0.01	–	3
Methoxychlor	<0.05	–	3
Dieldrin	<0.01	–	3
Endrin	<0.01	–	3

	Mean ± Standard Deviation[b]	Range	Number of Samples
Telodrin	<0.01	–	3
Chlordane	<0.05	–	3
Toxaphene	<0.10	–	3
Estimated PCBs	<0.20	–	3
Ronnel	<0.01	–	3
Ethion	<0.02	–	3
Trithion	<0.05	–	3
Diazinon	<0.10	–	3
Methyl Chlorpyrifos	0.06 ± 0.033	0.02–0.08	3
Methyl Parathion	<0.02	–	3
Ethyl Parathion	<0.02	–	3
Malathion	0.034 ± 0.016	0.02–0.051	3
Endosulfan I	<0.01	–	3
Endosulfan II	<0.01	–	3
Endosulfan Sulfate	<0.03	–	3

CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride.
[a]All samples were irradiated.
[b]For values less than the limit of detection, the detection limit is given as the mean.
[c]Sources of contamination: alfalfa, grains, and fish meal.
[d]Sources of contamination: soy oil and fish meal.
[e]All values were corrected for percent recovery.

Appendix E. Sentinel Animal Program

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Tables

E.1. Methods

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of test compounds. Under this program, the disease state of the rodents is monitored via sera or feces from extra (sentinel) or dose animals in the study rooms. The sentinel animals and the study animals are subject to identical environmental conditions. Furthermore, the sentinel animals come from the same production source and weanling groups as the animals used for the studies of test compounds.

For the toxicity studies of three perfluoroalkyl sulfonates, blood samples from the sentinel animals were collected and allowed to clot, and the serum was separated. All samples were processed appropriately and tested at the Research Animal Diagnostic Laboratory (RADIL, currently IDEXX BioResearch), University of Missouri (Columbia, MO), for determination of the presence of pathogens. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the study are also listed.

Blood was collected from five rats per sex.

Table E-1. Laboratory Methods and Agents Tested for in the Sentinel Animal Program

Method and Test	Time of Collection
Multiplex Fluorescent Immunoassay	
H-1 (Toolan's H-1 Virus)	End of quarantine
KRV (Kilham Rat Virus)	End of quarantine
Mycoplasma pulmonis	End of quarantine
PVM (Pneumonia Virus of Mice)	End of quarantine
RCV/SDA (Rat Coronavirus/Sialodacryoadenitis Virus)	End of quarantine
RMV (Rat Minute Virus)	End of quarantine
RPV (Rat Parvovirus)	End of quarantine
RTV (Rat Theilovirus)	End of quarantine
Sendai	End of quarantine
TMEV (Theiler's Murine Encephalomyelitis Virus)	End of quarantine

E.2. Results

All test results were negative.

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